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Emily Claire Liefoghe BSc(Hons)

**Studies on the Specificities of *In Vivo* and  
*In Vitro* Bioassays Used in the Potency  
Determination of Therapeutic Erythropoietin**

A thesis submitted to the Open University for the degree of  
Doctor of Philosophy

2006

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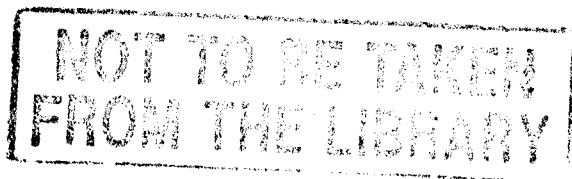
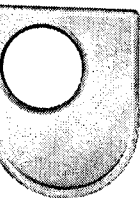
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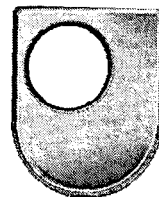
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## Abstract

Erythropoietin (Epo), a kidney-produced cytokine, promotes the proliferation and differentiation of erythroid progenitors into mature red blood cells.

The *in vivo* biological activity of Epo is dependent on it being adequately glycosylated. Glycosylation is a variable process and its precise effect on clearance mechanisms within the body has yet to be fully established. Studies have shown, however, that the loss of the terminal sialic acid residue, leading to exposure of the penultimate galactose residue, results in a dramatic reduction in activity, owing to the liver's galactose-dependent clearing action. Current *in vitro* cell-based bioassays for Epo are unaffected by such a phenomenon and therefore do not correlate with the *in vivo* bioassays.

As part of this project, strategies were devised for rendering cell-based bioassays sensitive to sialylation. The strategies' common feature was the inclusion of a mechanism for depleting or sequestering desialylated Epo via a galactose-binding protein (derived from either plants or the human liver). The assays were tested using a range of Epo samples desialylated to various degrees by neuraminidase treatment. This provided proof of principle that rational manipulation of *in vitro* bioassays rendering them sensitive to a factor known to affect *in vivo* activity can allow the prediction of bioactivity without the use of live animals.

Two Epo sample sets were investigated in addition to the neuraminidase-treated sample set - one produced by different culture and purification procedures, the other consisting of a batch of pharmaceutical-grade Epo separated into fractions by ion-exchange HPLC. Investigations were carried out by means of the most sensitive assay developed as well as a range of biological, biochemical and physico-chemical

techniques with a view to producing an analytical and comparative study. The results suggested that the relationship between galactose exposure and *in vivo* biological activity might not be as straightforward as had been initially thought.

# Table of Contents

<b>Table of Contents .....</b>	<b>i</b>
<b>List of Figures .....</b>	<b>ix</b>
<b>List of Tables .....</b>	<b>xiv</b>
<b>List of Tables .....</b>	<b>xiv</b>
<b>Abbreviations .....</b>	<b>xvi</b>
<b>Acknowledgements.....</b>	<b>xix</b>
<b>Publication arising from this thesis .....</b>	<b>xx</b>
<b>Chapter 1. Introduction.....</b>	<b>1</b>
1.1. Background.....	1
1.2. Structure of Epo .....	2
1.3. Site of Epo production .....	3
1.4. Erythropoiesis .....	8
1.5. O <sub>2</sub> sensing and transcription control .....	8
1.6. The Epo receptor and its signalling pathways.....	11
1.7. The history of recombinant Epo and its medical applications .....	14
1.8. Biologicals and batch testing .....	18
1.9. The post-hypoxic polycythaemic mouse bioassay.....	19
1.10. The normocythaemic mouse bioassay .....	20
1.11. Ethical issues.....	22
1.11.1. The suitability of replacing <i>in vitro</i> with <i>in vivo</i> assays .....	23

1.12. Clearance mechanisms.....	24
1.12.1. Hepatic clearance of desialylated glycoproteins.....	24
1.12.2. Renal clearance.....	27
1.12.3. Receptor-mediated clearance.....	28
1.12.4. Additional roles of sugars.....	29
1.13. Structure of the ASGP-R .....	30
1.14. H1 to H2 binding ratios.....	31
1.15. ASGP-R binding properties .....	34
1.16. Objectives .....	36
<b>Chapter 2. Materials and Methods.....</b>	<b>38</b>
2.1. Molecular Biology Techniques.....	38
2.1.1. Messenger RNA purification.....	38
2.1.2. First-strand cDNA synthesis.....	39
2.1.3. Polymerase chain reaction .....	39
2.1.3.1. Design of primers.....	39
2.1.3.2. PCR mix.....	40
2.1.4. TA cloning.....	42
2.1.5. Bacterial transformation .....	42
2.1.6. Blue/white screening .....	44
2.1.7. Preparation of DNA from transformed <i>E. coli</i> .....	45
2.1.8. Restriction endonuclease digestion plasmid DNA .....	46
2.1.9. Electrophoresis of DNA .....	46
2.1.10. Nucleotide sequencing.....	47
2.1.11. Purification of DNA from agarose gels .....	47
2.1.12. Dephosphorylation of plasmid DNA .....	48
2.1.13. Ligation of DNA fragments into a plasmid .....	48
2.1.14. Site-directed mutagenesis (SDM).....	49

2.1.15. Endotoxin-free large-scale preparation of DNA.....	52
2.1.16. Determination of DNA concentration and purity .....	52
2.2. Cell culture techniques.....	53
2.2.1. The Epo-dependent erythroleukaemic AS-E2 cell line .....	53
2.2.2. Maintenance of cell lines .....	53
2.2.2.1. Tissue culture reagents .....	53
2.2.2.2. Maintenance and culture of cell lines .....	54
2.2.2.3. Storage and recovery of cells .....	54
2.2.3. Transfections .....	54
2.2.3.1. Method for transfecting AS-E2 cells.....	55
2.2.3.2. Vector used for obtaining stable transfections - pIRESblast.....	55
2.2.3.3. Blasticidin selection .....	56
2.2.3.4. PCR-amplification from cell extract .....	58
2.2.4. AS-E2 Epo bioassays.....	59
2.2.4.1. Statistical analysis .....	60
2.3. Protein Analysis .....	60
2.3.1. Preparation of cell lysates for Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis .....	60
2.3.2. Measurement of protein content and preparation of samples for electrophoresis .	61
2.3.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (adapted from Laemmli, 1970) .....	61
2.3.4. Protein transfer .....	62
2.3.5. Western blotting .....	62
2.3.6. Isoelectric focusing.....	63
2.3.7. Silver staining .....	64
2.3.8. Protein transfer (specific to IEF gels).....	65
2.3.9. Densitometric analysis.....	65
2.3.10. Desialylation of Epo .....	65

2.3.11. Ion-exchange high performance liquid chromatography (HPLC) .....	66
2.3.12. Enzyme-linked immunosorbent assay (ELISA) .....	66
2.3.13. Fluorescence microscopy.....	67
2.3.14. Glycoanalysis (carried out by Dr Yuen, NIBSC) .....	69
2.4. In vivo bioassays: the post-hypoxic polycythaemic and normocythaemic mouse assays for Epo .....	69
2.4.1. The post-hypoxic polycythaemic mouse assay for Epo.....	69
2.4.2. The normocythaemic mouse assay for Epo .....	71
2.4.2.1. Statistical analysis of in vivo results .....	71
<b>Chapter 3. A sialylation-sensitive cell-based <i>in vitro</i> bioassay for erythropoietin; incorporation of the galactose-binding <i>Erythrina crista-galli</i> lectin. ....</b>	<b>73</b>
3.1. Introduction and aims of the study.....	74
3.2. Experimental procedures.....	79
3.2.1. Modified AS-E2 Epo bioassay incorporating the use of agarose-conjugated <i>Erythrina crista-galli</i> lectin (AS-E2/ECL bioassay) .....	79
3.2.2. Plate plan .....	79
3.2.3. <i>In vivo</i> mouse assay for Epo .....	81
3.2.4. Glycoanalysis.....	81
3.3. Results.....	81
3.3.1. Sample set 1: neuraminidase-desialylated Epo.....	81
3.3.1.1. Desialylation of Epo.....	81
3.3.1.2. In vivo post-hypoxic polycythaemic mouse assay .....	85
3.3.1.3. Unmodified AS-E2 bioassay .....	85
3.3.1.4. AS-E2/ECL bioassay.....	87
3.3.1.5. AS-E2/RCA <sub>120</sub> assay.....	92
3.3.1.6. Depletion of Epo was lectin-dependent.....	96
3.3.1.7. Lectin free in solution does not result in depletion of Epo.....	96



3.3.1.8. Comparison of the AS-E2, AS-E2/ECL and the in vivo mouse bioassays ....	99
3.3.2. Sample set 2: Range of pharmaceutical Epo preparations generated by different production and purification procedures .....	103
3.3.2.1. Background information.....	103
3.3.2.2. Unmodified AS-E2 bioassay .....	104
3.3.2.3. AS-E2/ECL bioassay.....	110
3.3.2.4. Comparison of the AS-E2, AS-E2/ECL, in vivo mouse and IEF assay .....	110
3.3.3. Sample set 3: pharmaceutical Epo fractionated by anion-exchange HPLC .....	115
3.3.3.1. Background on the anion-exchange fractionated samples .....	115
3.3.3.2. Isoelectric focusing .....	115
3.3.3.3. In vivo mouse assays for Epo.....	119
3.3.3.4. Glycoanalysis .....	124
3.3.3.5. Unmodified AS-E2 and AS-E2/ECL bioassays .....	131
3.3.3.6. Correlation of in vivo and in vitro bioactivity with non-biological data (IEF and glycan mapping) .....	136
3.4. Discussion .....	139
3.5. Conclusion .....	145

## **Chapter 4. Development of a sialylation-sensitive cell-based bioassay; stable transfection of an Epo-dependent cell line with the asialoglycoprotein receptor ..... 147**

4.1. Introduction and aims of the study.....	147
4.2. Experimental procedures.....	152
4.2.1. PCR-amplification of first-strand cDNA and cloning of the ASGP-R.....	152
4.2.2. Identification of the H2b splice variant .....	153
4.2.3. Plasmid construction.....	156
4.2.4. Expression of immunoactive ASGP-Rs: Western blotting.....	158

4.2.5. Expression of functional ASGP-R: binding of fluorophore-labelled asialo- $\alpha$ -1-acid glycoprotein .....	158
4.2.6. Bioassays .....	158
4.2.7. Plate plan .....	158
4.3. Results.....	160
4.3.1. Verification of p[H1-IRES-H2b]IRESblast by sequencing.....	160
4.3.2. The H2b splice variant was identified using analytical primers.....	160
4.3.3. Screening of transfection reagents.....	166
4.3.4. Expression of the ASGP-R in AS-E2 cells.....	166
4.3.5. Fluorescence Microscopy .....	169
4.3.6. Bioassays .....	173
4.3.6.1. Evaluating sensitivity to desialylation in the ASGP-R-transfected AS-E2 cell line.....	173
4.3.6.2. The effect of asialo- $\alpha$ -1-acid glycoprotein.....	178
4.4. Discussion .....	184
4.4.1. Optimisation of stable transfection protocol.....	184
4.4.2. The Internal Ribosome Entry Site (IRES).....	185
4.4.3. Putative new splice variant .....	188
4.4.4. Soluble receptors .....	188
4.4.5. Limitations of using the ASGP-R.....	189
4.5. Conclusion .....	190

## **Chapter 5. Development of a sialylation-sensitive cell-based bioassay; genetic manipulation of an Epo-dependent cell line to display cell-surface**

<b>galactose-specific plant lectins .....</b>	<b>191</b>
5.1. Introduction and aims of the study.....	191
5.1.1. Advantages of plant lectins over the ASGP-R .....	192
5.1.2. <i>Erythrina crista-galli</i> lectin .....	193

5.1.3. The <i>Ricinus communis</i> lectins .....	194
5.1.4. Designing chimeric decoy receptors.....	196
5.2. Experimental Procedures .....	200
5.2.1. RNA isolation and first-strand cDNA synthesis.....	200
5.2.2. PCR-amplification of galactose-binding plant lectins .....	201
5.2.2.1. Site-directed mutagenesis (SDM) .....	201
5.2.3. Introduction of a 5' Epo receptor signal peptide by PCR.....	204
5.2.4. PCR-amplification of the Epo-R transmembrane domain.....	207
5.2.5. Assembly of the plant lectin/Epo-R chimeric construct and ligation into pIRESblast.....	209
5.2.6. Replacement of the Epo-R transmembrane domain-encoding region with that of the growth hormone receptor transmembrane and degradation domains .....	209
5.2.7. Establishment of AS-E2 cells stably transfected with plant lectin/TM-expressing vector constructs.....	214
5.2.8. Bioassays .....	214
5.2.9. Plate plan .....	214
5.3. Results.....	215
5.3.1. AS-E2 cells engineered to display ECL at the cell surface (ECL-TM) were not viable .....	215
5.3.2. Survival of AS-E2 cells stably transfected with RCA <sub>60</sub> -TM and RCA <sub>120</sub> -TM and retention of Epo-dependency.....	215
5.3.3. Screening of stably transfected cell lines.....	215
5.3.4. Fluorescence microscopy.....	218
5.3.5. Bioassays .....	221
5.3.6. PCR-amplification from cell extracts .....	226
5.4. Discussion.....	229
5.5. Conclusion .....	233

<b>Chapter 6. Discussion .....</b>	<b>234</b>
6.1. Epo as a pharmaceutical analysis problem .....	234
6.2. Control specifications for Epo .....	235
6.3. Factors determining the <i>in vivo</i> biological activity of Epo .....	236
6.4. Exposed galactosyl residues as a potential analytical target.....	236
6.4.1. <i>In vitro</i> bioassays as analytical tools .....	238
6.4.2. Incorporation of galactosyl-binding into the <i>in vitro</i> bioassay .....	239
6.4.2.1. Proposed strategy for increasing ASGP-R expression .....	240
6.4.2.2. Sialylation and <i>in vitro</i> activity .....	242
6.4.2.3. The viability of targeting the galactosyl residue .....	244
6.4.3. Non-biological approaches to identifying activity-determining features .....	245
6.4.3.1. The utility of charge-based separations.....	245
6.4.3.2. The utility of glycan mapping .....	246
6.5. Conclusions, future developments and specification recommendations.....	249
 <b>Chapter 7. Summary.....</b>	 <b>251</b>
 <b>References .....</b>	 <b>255</b>
 <b>Appendix.....</b>	 <b>295</b>

## List of Figures

Fig. 1.2.1. Schematic representation of the primary structure of human Erythropoietin .....	4
Fig. 1.2.2. Ribbon model of erythropoietin	
Fig. 1.2.3. Schematic representation of an N-linked sugar chain of the complex type and .....	5
Fig. 1.2.3. Schematic representation of an N-linked sugar chain of the complex type and .....	6
Fig. 1.4.1. Schematic representation of the development stages of erythroid progenitors. ....	9
Fig. 1.6.1. Structure of the human Epo receptor dimer and its associated signalling molecules. ....	15
Fig. 1.13.1. Schematic representation of the two subunits of the asialoglycoprotein receptor (ASGP-R).....	32
Fig. 2.1.1. Diagram of the pGEM-T vector (Promega).....	43
Fig. 2.1.2. Restriction endonuclease analysis of small scale preparation of plasmid DNA aimed at establishing whether mutation by SDM had been successful.....	51
Fig. 2.2.1. Map of pIRESblast vector used in mammalian transfections.....	57
Fig. 3.1.1. Schematic representation of the rationale behind the AS-E2/ECL bioassay protocol. ....	77
Fig. 3.2.1. Plate plan for cell-based Epo bioassays.....	80
Fig. 3.3.1. Epo treated with neuraminidase for increasing lengths of time subjected to isoelectric focusing gel electrophoresis followed by western blot with anti-Epo antibody.....	82

Fig. 3.3.2. Proliferative response of AS-E2 cells to untreated and neuraminidase-treated Epo. ....	88
Fig. 3.3.3. Proliferative response of AS-E2 cells to untreated and neuraminidase-treated Epo subsequent to incubation with agarose-conjugated Erythrina crista-galli lectin (ECL).....	90
Fig. 3.3.4. Proliferative response of AS-E2 cells to untreated and 20 minute-neuraminidase-treated Epo subsequent to incubation with agarose-conjugated ECL. ....	93
Fig. 3.3.5. Proliferative response of AS-E2 cells to untreated and neuraminidase-treated Epo subsequent to incubation with agarose-conjugated RCA <sub>120</sub> . ....	95
Fig. 3.3.6. Proliferative response of AS-E2 cells to untreated and neuraminidase-treated Epo subsequent to incubation with agarose alone.....	97
Fig. 3.3.7. Proliferative response of AS-E2 cells to neuraminidase-treated Epo (sample t=1800 min) subsequent to incubation with agarose-conjugated ECL with or without the presence of lactose. ....	98
Fig. 3.3.8. Effect of Erythrina crista-galli lectin free in solution on the proliferative response of AS-E2 cells to untreated and neuraminidase-treated Epo. ....	100
Fig. 3.3.9. Comparison of in vivo, AS-E2 and AS-E2/ECL bioassays.....	101
Fig. 3.3.10. Isoelectric focusing of the four pharmaceutical Epo preparations detected by Western blotting: Gr, SpB, M2G and L2. ....	105
Fig. 3.3.11. Proliferative response of AS-E2 cells to the four pharmaceutical Epo preparations.....	108
Fig. 3.3.12. Proliferative response of AS-E2 cells to the four pharmaceutical Epo preparations subsequent to incubation with agarose-conjugated ECL. ....	111
Fig. 3.3.13. Comparison of in vivo, AS-E2 and AS-E2/ECL bioassays for the four pharmaceutical Epo preparations. ....	113

Fig. 3.3.14. HPLC UV trace of pharmaceutical-grade Epo (Amgen) eluted from an anionic exchange column (MonoQ).....	116
Fig. 3.3.15. Isoelectric focusing gel of ion-exchange HPLC-separated fractions of a batch of pharmaceutical-grade Epo (Amgen). ....	117
Fig. 3.3.16. Estimates of the specific activities of the four ion-exchange fractionated Epo samples by in vivo post-hypoxic polycythaemic mouse assay (table and graph). ....	120
Fig. 3.3.17. Estimates of the specific activities of the four ion-exchange fractionated Epo samples by in vivo normocythaemic mouse assay (table and graph).....	122
Fig. 3.3.18. Glycan profiles of ion-exchange HPLC-separated Epo Fractions A to D. ....	126
Fig. 3.3.19. Proliferative response of AS-E2 cells to Epo Fractions A to D by unmodified AS-E2 bioassay and by AS-E2/ECL bioassay. ....	133
Fig. 4.1.1. Schematic representation of the decoy receptor strategy. ....	149
Fig. 4.2.1. PCR amplification of ASGP-R-H1 and -H2.....	154
Fig. 4.2.2. PCR amplification of [ASGP-R-H1]-[IRES]-[ASGP-R-H2b] with EcoRI and ClaI restriction ends. ....	157
Fig. 4.2.3. Plate plan for stably transfected cell-based Epo bioassay. ....	159
Fig. 4.3.1. The design of the intended vector and the primers used for verification by sequencing.....	161
Fig. 4.3.2. Asialoglycoprotein receptor H2 subunit sequence, showing the different possible splice variants and primers used for their identification.....	162
Fig. 4.3.3. Splice variant identification of ASGP-R-H2 clones.....	164
Fig. 4.3.4. SDS-PAGE/Western blot of ASGP-R-H1 expressed recombinantly in AS-E2 cells and endogenously in Hep-G2 cells, using an anti-ASGP-R antibody...	167

Fig. 4.3.5. Expression of functional ASGP-R by ASGP-R-transfected AS-E2 cells: binding of fluorophore-labelled asialo- $\alpha$ -1-acid glycoprotein. ....	171
Fig. 4.3.6. Proliferative response of empty vector-transfected AS-E2 cells and AS-E2 cells stably transfected with the asialoglycoprotein receptor H1 and H2b subunits to both intact and the most extensively desialylated Epo (samples $t=0$ min and $t=1800$ min. ....	174
Fig. 4.3.7. Proliferative response of empty vector- transfected AS-E2 cells and AS-E2 cells stably transfected with the asialoglycoprotein receptor H1 and H2b subunits to both intact and mid-desialylated Epo (samples $t=0$ min and $t=480$ min). ....	176
Fig. 4.3.8. Proliferative response of empty vector-transfected AS-E2 cells and AS-E2 cells stably transfected with the asialoglycoprotein receptor H1 and H2b subunits to both intact and slightly desialylated Epo (samples $t=0$ min and $t=20$ min). ....	179
Fig. 4.3.9. Proliferative response of empty vector- transfected AS-E2 cells and AS-E2 cells stably transfected with the asialoglycoprotein receptor H1 and H2b subunits to both intact and desialylated Epo (samples $t=0$ min and $t=1800$ min) in the presence of intact $\alpha$ -1-acid glycoprotein (AAGP) or asialo-AAGP. ....	182
Fig. 4.4.1. Schematic representation of the IRES cassette at DNA and mRNA levels. .....	187
Fig. 5.1.1. Schematic representation of the design of a surface-expressed decoy receptor incorporating a galactose-binding plant lectin domain. ....	197
Fig. 5.2.1. PCR amplification of <i>Erythrina crista-galli</i> lectin. ....	202
Fig. 5.2.2. PCR amplification of the RCA <sub>60</sub> and RCA <sub>120</sub> B chains. ....	203
Fig. 5.2.3. PCR amplification of plant lectins (ECL, RCA <sub>60</sub> and RCA <sub>120</sub> B chains) with incorporation of the Epo-R signal peptide. ....	206



Fig. 5.2.4. PCR amplification of the Epo-R transmembrane domain incorporating a 5' flexible linker. ....	208
Fig. 5.2.5. Schematic representation of the sub-cloning strategy for constructing a mammalian expression vector encoding a transmembrane domain-anchored plant lectin. ....	210
Fig. 5.2.6. PCR amplification of the Growth Hormone Receptor mutant (4Pro→Ala) transmembrane/internalisation domain. ....	213
Fig. 5.3.1. SDS-PAGE/Western blot using an antibody specific for both RCA <sub>60</sub> and RCA <sub>120</sub> to investigate whether AS-E2 cells stably transfected with the p[RCA <sub>60/120</sub> -Epo-R TM]IRESblast vectors were successfully expressing RCA <sub>60</sub> or RCA <sub>120</sub> B chain. ....	216
Fig. 5.3.2. Investigating whether expression of a functional galactose-binding chimeric protein had been achieved in lectin display vector-transfected AS-E2 cells: binding of fluorophore-labelled asialo- $\alpha$ -1-acid glycoprotein. ....	219
Fig. 5.3.3. Proliferative response of AS-E2 cells stably transfected with the empty pIRESblast, pIRESblast[RCA <sub>60</sub> -Epo-R TM] and pIRESblast[RCA <sub>120</sub> -Epo-R TM] vectors to intact and extensively desialylated Epo (samples t=0 min and t=1800 min). ....	222
Fig. 5.3.4. Proliferative response of untransfected AS-E2 cells and AS-E2 cells stably transfected with the RCA <sub>60</sub> or RCA <sub>120</sub> B chain gene fused to the transmembrane domain of the mutant growth hormone receptor (activation disabled but internalisation competent) to intact and extensively desialylated Epo (samples t=0 min and t=1800 min). ....	224
Fig. 5.3.5. PCR amplification of RCA (expected product size= $\sim$ 780 bp) and actin (expected product size= $\sim$ 630 bp) from cell extracts. ....	227

## List of Tables

Table 3.1.1. Commonly used lectins, the plant species from which they are derived and their binding specificities. ....	75
Table. 3.3.1. Estimates of the specific activities of the untreated and neuraminidase-treated Epo samples. ....	86
Table 3.3.2. Calculation of the mean pI values of the four pharmaceutical Epo preparations. ....	105
Table. 3.3.3. Estimates of the specific activities of the four pharmaceutical Epo preparations. ....	107
Table 3.3.4. Table of mean pI values and pI values of the strongest band of the ion-exchange HPLC-separated Epo Fractions. ....	117
Table 3.3.5. Table of percentages of each type of carbohydrate in Epo Fractions A to D. ....	127
Table 3.3.6. Table of the proportions of each type of saccharide or oligosaccharide structure in Epo Fractions A to D. ....	129
Table 3.3.7. Table of the combined estimate of potencies of Epo Fractions A to D by unmodified AS-E2 bioassay and by AS-E2/ECL bioassay. ....	132
Table 3.3.8. Measurement of association between the in vivo assays (polycythaemic and normocythaemic) and carbohydrate proportions identified by glycoanalysis, using Pearson correlations. ....	137
Table 3.3.9. Measurement of association between the in vitro assays (AS-E2 and AS-E2/ECL), in vivo assays (polycythaemic and normocythaemic) and carbohydrate proportions identified by glycoanalysis, using Pearson correlations. ....	140

Table 3.4.1. Methods that can detect changes in isoform distribution and an assessment of how sensitive they were when performing tests on each of the three Epo sample sets. .... 144

Table 4.3.1. Potencies of desialylated Epo (sample t=1800 min) relative to intact Epo (sample t=0 min) in the ASGP-R-transfected AS-E2 cell bioassay. .... 174

Table 4.3.2. Potencies of desialylated Epo (sample t=480 min) relative to intact Epo (sample t=0 min) in the ASGP-R-transfected AS-E2 cell bioassay. .... 176

Table 4.3.3. Potencies of desialylated Epo (sample t=20 min) relative to intact Epo (sample t=0 min) in the ASGP-R-transfected AS-E2 cell bioassay. .... 179

Table 6.1. Methods available (including cell-based assays developed during the course of this project) for detecting changes in isoform distribution and how sensitively they behaved when testing each of the three Epo sample sets..... 248

## Abbreviations

aa	amino acid
AAGP	alpha-1 acid glycoprotein
ASGP-R	asialoglycoprotein receptor
BFU-E	erythroid burst-forming unit
bp	base pair(s)
BSA	bovine serum albumin
CHO	Chinese hamster ovary
CFU-E	erythroid colony-forming unit
DHFR	dihydrofolate reductase
DMEM	Dulbecco's-modified Eagle's medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	<i>Erythrina crista-galli</i> lectin
ELISA	enzyme linked immunosorbent assay
EDTA	ethylenediamine tetra-acetic acid
EMP	erythropoietin mimetic peptide
EP	European Pharmacopoeia
Epo	erythropoietin
Epo-R	erythropoietin receptor
ER	endoplasmic reticulum
FCS	foetal calf serum
GH-R	growth hormone receptor

GS	glutamine synthetase
HPLC	high performance liquid chromatography
IEF	isoelectric focusing
IMDM	Iscove's Modified Dulbecco's Medium
IPTG	isopropyl $\beta$ -D-thiogalactoside
IRES	internal ribosome entry site
IU	International units
JAK	Janus kinase
Kb	kilo base(s)
KDa	Kilo Daltons
LB	Luria-Bertani broth
LBamp	Luria-Bertani broth containing 0.1 mg/ml ampicillin
M	DNA or protein marker
mA	milli amps
MAPK	mitogen-activated protein kinase
MCS	multiple cloning site
mRNA	messenger RNA
PCR	polymerase chain reaction
pI	isoelectric point
RCA <sub>60</sub>	Ricin
RCA <sub>120</sub>	<i>Ricinus communis</i> agglutinin
recHuEpo	recombinant human erythropoietin
rpm	revolutions per minute
RNA	ribonucleic acid

SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
s.e.m.	standard error of the mean
SH2	Src homology 2
SHP1	SH2-containing protein tyrosine phosphatase 1
STAT	signal transduction and activator of transcription
t= 0 min	Epo treated with sialidase for 0 minutes
Triton X-100	t-octylphenoxypolyethoxyethanol
Tween-20	Polyoxyethylene-sorbitan monolaurate
TBE	Tris-boric acid-EDTA buffer
TM	Transmembrane domain
UV	ultra violet
V	volts
X-Gal	5-Bromo-4-chloro-3-indolyl- $\beta$ -D- galactopyranoside

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the galactose-binding Erythrina crista-galli lectin. *Biologicals* **33**,161-7.



## **Chapter 1. Introduction**

### **1.1. Background**

The task of transporting oxygen from the lungs to tissue elsewhere in the body is performed by erythrocytes (red blood cells) via the high-affinity oxygen-binding molecule, haemoglobin. Since red blood cells have a finite lifespan – generally accepted as being 120 days, although this figure may have to be revised slightly in the light of recent research (Werre *et al*, 2004) – new ones have to be produced all the time.\* It is, however, essential to produce the right quantities, since excess production can make the blood become too viscous, with an attendant risk of high blood pressure, strokes and thrombosis (Anderson & Scotti, 1972), while underproduction can lead to the fatigue, shortness of breath and dizziness associated with anaemia (reviewed in Gabrilove, 2000). Erythropoiesis, the process whereby pluripotent haematopoietic stem cells within the bone marrow proliferate and differentiate into mature red blood cells in response to hypoxia (low oxygen levels), is stimulated by the glyco-hormone erythropoietin (Epo) (Gregory & Eaves, 1978; Bondurant & Koury, 1986; Erslev, 1953). Since it was cloned in 1985 (Jacobs *et al*, 1985; Lin *et al*, 1985) and subsequently produced on an industrial scale, Epo has become one of the most successful biopharmaceutical products to date, freeing chronic renal failure patients from having to undergo the repeated blood transfusions needed to treat the anaemia associated with end-stage renal disease.

In the case of Epo, and indeed any biological product, the physico-chemical, biological and immunochemical properties have to be fully characterised by procedures that conform to the guidelines set by the International Conference on

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\* Around 2.3 million per second (Lappin, 2003)

Harmonisation (1999). According to the European Pharmacopoeia (Fifth Edition, 2005), the only means of evaluating the true biological activity of Epo is to use *in vivo* tests on mice – measuring reticulocyte production or  $^{59}\text{Fe}$  incorporation into newly synthesised red blood cells. Although there is a drive to replace *in vivo* bioassays with assays that do not involve animals, this has not yet been achieved in the case of Epo, as potencies obtained by the two procedures do not correlate, in particular because of the role the liver-expressed asialoglycoprotein receptor plays in clearing incompletely sialylated glycoproteins from the circulation (Fukuda *et al*, 1989). Correct glycosylation is essential to the *in vivo* activity of Epo whereas it is more or less irrelevant to its *in vitro* activity as tested by immuno- or cell-based assays (Tsuda *et al*, 1990). Manipulating *in vitro* bioassays - thereby rendering them responsive to factors known to affect bioactivity - is one option with potential for predicting *in vivo* activity without the need to use live animals. This could be done by depleting Epo solutions of certain glycoforms prior to use in an Epo-dependent cell-based assay or by genetically engineering Epo-dependent cells to express selective decoy receptors selective. The work described in this thesis investigates this possibility.

## **1.2. Structure of Epo**

Human Epo is synthesised as a 193-amino acid polypeptide. A 27-amino acid signal sequence and a carboxy-terminal arginine residue are removed during processing to produce a 165-amino acid mature protein with a molecular mass of 30.4kDa (reviewed in Moritz *et al*, 1997). It has a compact globular structure consisting of four amphipathic  $\alpha$ -helical bundles (Wen *et al*, 1994; Elliott *et al*, 1997). There are two disulfide bonds, between cysteines 29 and 33 and cysteines 7 and 161 respectively, the latter bond being highly conserved across species owing to its critical

role in stabilising the protein and maintaining it in the correct shape for receptor binding (Wen *et al*, 1993; Elliott *et al*, 1996).

Sugar chains account for approximately 40% of Epo's apparent molecular weight (unglycosylated Epo is 18 kDa). Epo is N-glycosylated at asparagines 24, 38 and 83 while serine 126 is O-glycosylated. The N-glycan chains of Epo are of the complex type (see Fig. 1.2.1. for a schematic representation of the primary structure of human Epo, and Fig. 1.2.2. for a ribbon model).

Oligosaccharide chains can differ in a variety of ways:

- by type of glycosylation: glycosylated on asparagine (N-glycosylation) or on serine or threonine residues (O-glycosylation);
- by degree of branching: mono-antennary, bi-antennary, tri-antennary, tetra-antennary (1, 2, 3 or 4 branches respectively);
- by level of sialylation: unsialylated, monosialylated, disialylated, trisialylated, tetrasialylated;
- in terms of fucosylation, the presence of polylactosamines, etc.

(see Fig. 1.2.3. for a description of the various types of glycosylation.)

### **1.3. Site of Epo production**

In adults, Epo is produced by a population of interstitial cells in the cortex of the kidney (Maxwell *et al*, 1993) and circulates in the blood to stimulate erythropoiesis in the bone marrow. In the foetus, Epo is produced by liver parenchymal cells, but soon after birth production switches to the kidneys (Zanjani *et al*, 1977).

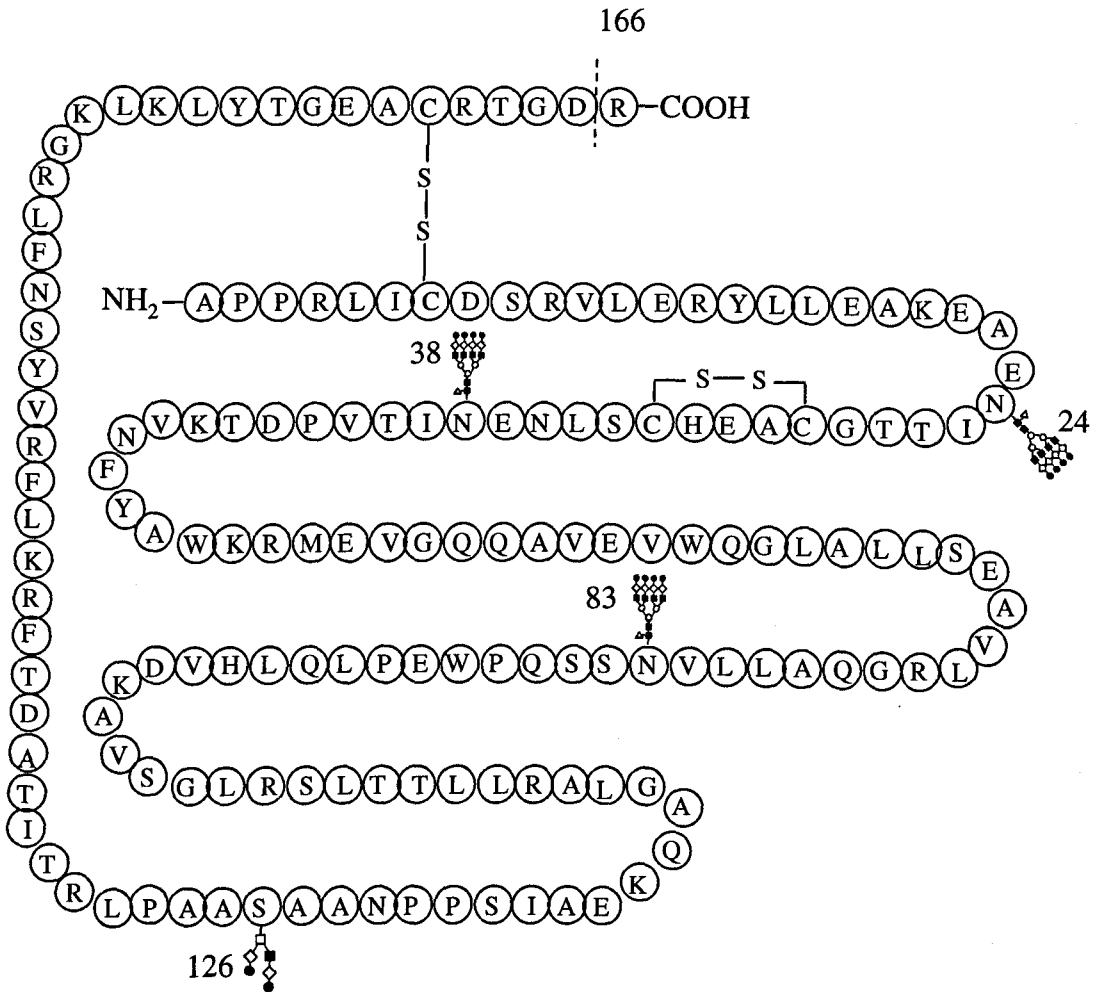




Fig. 1.2.1. Schematic representation of the primary structure of human Erythropoietin.

Arginine 166 is cleaved during post-translational modification, as is a 27-amino acid signal peptide (not shown), to leave a 165-amino acid mature glycoprotein.

There are two disulfide bonds, one between cysteines 29 and 33, the other between cysteines 7 and 161. Asparagines 24, 38 and 83 are N-glycosylated and serine 126 is O-glycosylated, indicated by  and  sugar chains respectively

(modified from Jelkmann (1992)).

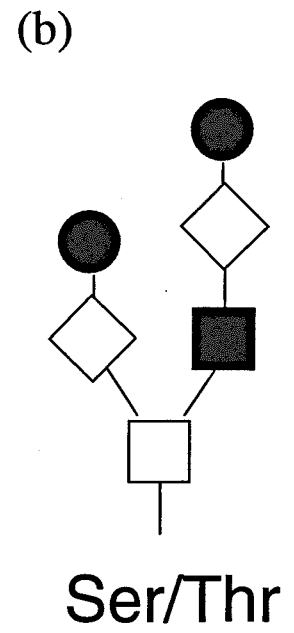
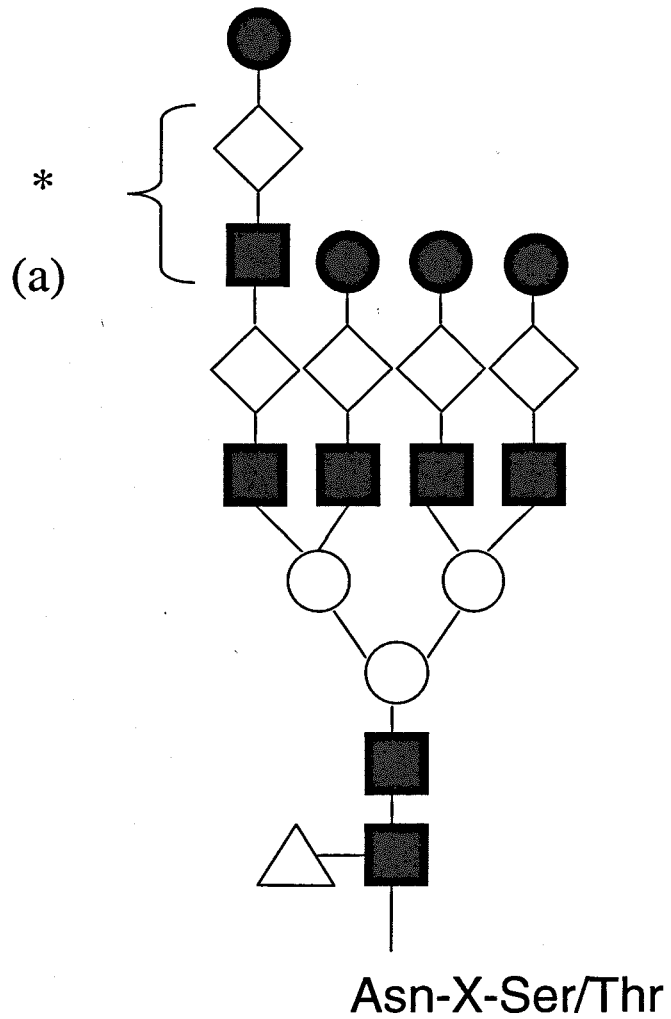




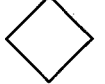



Fig. 1.2.2. Ribbon model of erythropoietin; image of structure of erythropoietin (PDB code 1BUY) based on NMR structure published by Cheetham *et al*, 1998 ([http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/genfigs/RunGenfigs.pl?call\\_type=existingpdb&pdbcode=1buy](http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/genfigs/RunGenfigs.pl?call_type=existingpdb&pdbcode=1buy)).

Fig. 1.2.3. (Facing page) (a) Schematic representation of an N-linked sugar chain of the complex type. The consensus site for N-glycosylation is Asn-X-Ser/Thr, where X can be any amino acid except Pro or Asp. The sugar may have four branches (tetra-antennary), three branches (tri-antennary), two branches (bi-antennary) or just one branch (mono-antennary). The illustration shows a tetra-antennary structure. Sialic acids may or may not be present. Extended polylactosaminyl structures (denoted by an asterisk) may be formed by additional [Galactose-N-acetylglucosamine] moieties. N-acetylgalactosamine-sulphate sometimes replaces the galactose-sialic acid moiety.

(b) Schematic representation of an O-linked bi-antennary sugar chain.

Figs (a) and (b) modified from Lowe and Marth, 2003.



- Legend:
-  N-acetylglucosamine
  -  Mannose
  -  Galactose
  -  Sialic acid +/-
  -  Fucose
  -  N-acetylgalactosamine

### **1.4. Erythropoiesis**

In adult bone marrow, pluripotent haematopoietic stem cells develop into erythroid progenitors (see Fig. 1.4.1. for a schematic representation of the various stages).

The earliest erythroid-progenitors, burst-forming unit-erythroids (BFU-Es), mature in the presence of interleukin-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and stem cell factor (SCF), and begin to express erythropoietin receptors (Epo-R), consequently gaining the ability to respond weakly to Epo (Emerson *et al*, 1988; Dai *et al*, 1991). After a few days, colony-forming erythroid units (CFU-E) develop which are highly responsive to Epo. These go on to become erythroblasts, by which time Epo sensitivity and the number of Epo receptors have declined until there is no longer a dependence on Epo (Gregory & Eaves, 1978; Gregory & Eaves, 1977). During erythropoiesis, calcium uptake by the cells increases, the Epo-R and other intracellular signalling proteins become phosphorylated, haemoglobin accumulates and, lastly, enucleation (exclusion of the nucleus) takes place (Mladenovic & Kay, 1988; Dusanter-Fourt *et al*, 1992; Quelle & Wojchowski, 1991; Koury *et al*, 1987). This is the series of events that occurs in the presence of Epo. Physiological concentrations of Epo are generally low, however, and if Epo does not bind its receptor, the cells undergo apoptosis (Muta & Krantz, 1993).

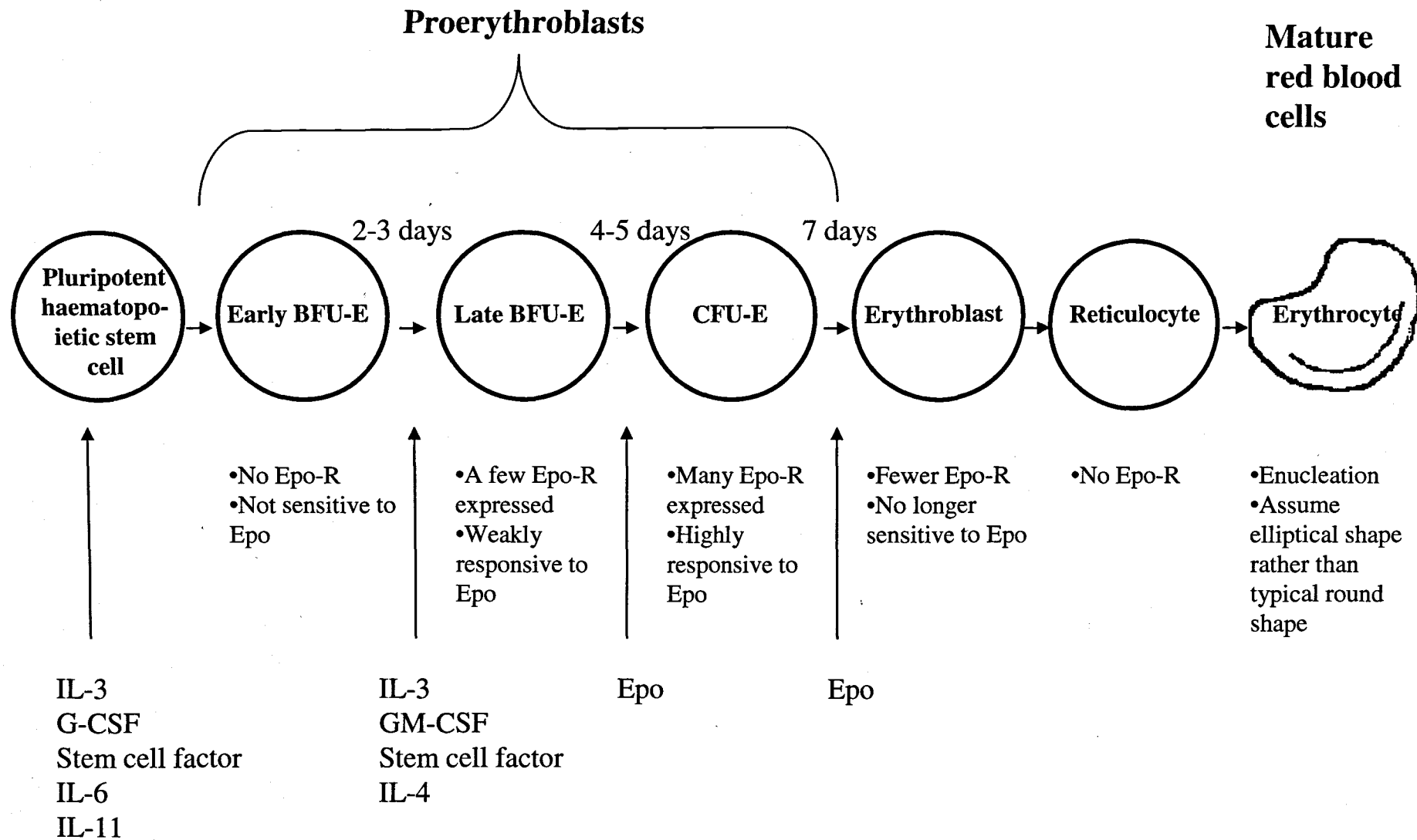
### **1.5. O<sub>2</sub> sensing and transcription control**

If there is a reduction in the O<sub>2</sub> capacity of the blood (i.e. a fall in haemoglobin concentration), a decrease in arterial pO<sub>2</sub> (e.g. at high altitudes), or an increase in the blood's O<sub>2</sub> affinity (for example, due to a mutant haemoglobin or an increase in blood alkalinity), Epo gene expression is triggered (Miller *et al*, 1982; Lenfant & Sullivan, 1971; Hebbel *et al*, 1978; Miller *et al*, 1973). The hypoxia-inducible factor (HIF)-1



Fig. 1.4.1. (Facing page) Schematic representation of the development stages of erythroid progenitors.

Burst-forming unit erythroids (BFU-Es) are less responsive to Epo and have few Epo receptors (thus requiring higher Epo concentrations for their amplification), but their numbers increase as the BFU-E matures into the colony-forming unit erythroid (CFU-E). Since they feature the highest number of Epo receptors, CFU-Es are more sensitive to Epo and require lower Epo concentrations for differentiation into reticulocytes and, ultimately, mature erythrocytes. The Epo receptors are no longer present in reticulocytes and mature erythrocytes (adapted from Fisher, 2003).



plays an integral role in this process. HIF-1 is a dimer consisting of an  $\alpha$ - and a  $\beta$ -subunit. The  $\alpha$ -subunit contains a C-terminal transactivation domain, whereas the  $\beta$ -subunit is a transcription factor identical in sequence to the aryl-hydrocarbon nuclear translocator (ARNT). Messenger RNA (mRNA) encoding these subunits remains constant, whether or not the body is experiencing hypoxia. Protein levels of the  $\beta$ -subunit also remain constant, but HIF-1 $\alpha$  is unstable when oxygen levels are normal as it contains two oxygen-dependent proteolytic degradation domains. If the oxygen concentration is above a certain level in the cell, HIF-1 $\alpha$  is hydroxylated at prolines 402 and 564, enabling binding to the von-Hippel-Lindau protein (pVHL), which in turn mediates the formation of a complex with E3 ubiquitin ligase. The final step is degradation by the proteasome. Under hypoxic conditions, the HIF-1 $\alpha$  evades degradation, enters the nucleus, dimerises with HIF-1 $\beta$  and binds the Hypoxia Response Element (HRE) (reviewed in Jelkmann, 2004).

### **1.6. The Epo receptor and its signalling pathways**

The Epo-R shares homology with receptors for Interleukin (IL)-3, IL-4, IL-6, IL-7, granulocyte and macrophage colony-stimulating factor, granulocyte colony-stimulating factor, growth hormone and prolactin (Finidori & Kelly, 1995), all of which are members of the cytokine class I receptor superfamily (Bazan, 1990). The class II receptor family contains the less structurally related receptors for interleukin-10 and interferons  $\alpha$ ,  $\beta$ , and  $\lambda$  (Kelly *et al*, 1993).

The human Epo-R was first cloned in 1991 (Ehrenman & St John, 1991; Maouche *et al*, 1991; Noguchi *et al*, 1991). The mature form of the Epo-R is a 483 amino acid (aa) long (508 aa when the signal peptide is uncleaved) single transmembrane-spanning glycoprotein receptor with a molecular weight of 66 kDa when glycosylated with

complex-type sugars (Yoshimura *et al*, 1990). Notable among the structural features of the Epo-R exoplasmic domain are its four conserved cysteine residues and the Trp-Ser-X-Trp-Ser (WSXWS) motif (where X is any amino acid, A in the case of the Epo-R) lying very close to the transmembrane domain. Site-directed mutations of the WSAWS motif have revealed it to be crucial to protein-folding, ligand-binding, and signal transduction (Yoshimura *et al*, 1992). As with many members of the cytokine receptor family, the membrane-proximal regions contain two conserved motifs, called box 1 and box 2. Box 1 is proline-rich, while box 2 contains hydrophobic residues followed by positively charged amino acids. Their presence is required for the induction of proliferation-promoting signals (Murakami *et al*, 1991; Narazaki *et al*, 1994).

The Epo-R is a pre-formed dimer (Livnah *et al*, 1999). The act of Epo binding to its receptor has been shown by X-ray crystallography (Syed *et al*, 1998) and nuclear magnetic resonance (Cheetham *et al*, 1998) to force the receptor to take on a new conformation that allows optimised transduction signalling. Chimeric forms of the Epo-R have been created in which its transmembrane domain was replaced by various types of coiled coils. These coils constrained the subunits of the dimer receptor into different orientations and demonstrated that certain conformations facilitate activation, whereas one corresponds to the inactive form of the receptor. Additionally, if Arg<sup>129</sup> in the extracellular domain of the Epo-R is changed to a cysteine, constitutive dimers arise, accompanied by constitutive proliferation (Watowich *et al*, 1992). One molecule of Epo binds two receptors at a time, one with high affinity and one with low affinity (Philo *et al*, 1996).

Unlike receptor tyrosine kinases, the Epo-R cytoplasmic domain contains no intrinsic tyrosine kinase activity and instead associates with a tyrosine kinase, Janus-activated

kinase 2 (Jak2) (Witthuhn *et al*, 1993). Conformational change of the two intracellular domains of a dimer initiates activation of the two associated Jak2 molecules. The activated Jak2 proteins tyrosine phosphorylate each other, the EpoR and other signalling molecules, creating docking sites for signalling proteins containing Src homology 2 (SH2) domains. Many proliferative and anti-apoptotic signal-transduction pathways get activated, including the phosphatidyl-inositol 3-kinase/Akt, Ras/mitogen-activated protein (MAP) kinase, phospholipase C-protein kinase C (PLC-PKC), and Signal-Transducer and Activator of Transcription 5 (STAT5) pathways (Damen *et al*, 1993; Damen *et al*, 1995a; Bao *et al*, 1999; Bao *et al*, 1999; Torti *et al*, 1992; Miura *et al*, 1994; Ren *et al*, 1994; Damen *et al*, 1995b). Following activation by phosphorylation, the cytoplasmic transcription factor STAT5 dimerizes and translocates to the nucleus, where it binds to the Bcl-x<sub>L</sub> promoter, thereby driving expression of this anti-apoptotic protein member of the Bcl family (Silva *et al*, 1999).

The maturation and proliferation pathways are distinct from the anti-apoptotic pathway. One line of evidence in support of this is the mutant cell-line J2E-NR, which has lost its ability to proliferate in the presence of Epo but does not undergo apoptosis (Tilbrook *et al*, 1996). Moreover, treatment of the murine Epo-dependent HCD57 cell line with orthovanadate, a tyrosine phosphatase inhibitor, has been shown to result in increased levels of tyrosine protein phosphorylation and prevention of apoptosis while leaving proliferation unaffected. Conversely, blocking the pro-proliferative MAP kinase pathway with a chemical inhibitor did not cause apoptosis (Lawson *et al*, 2000).

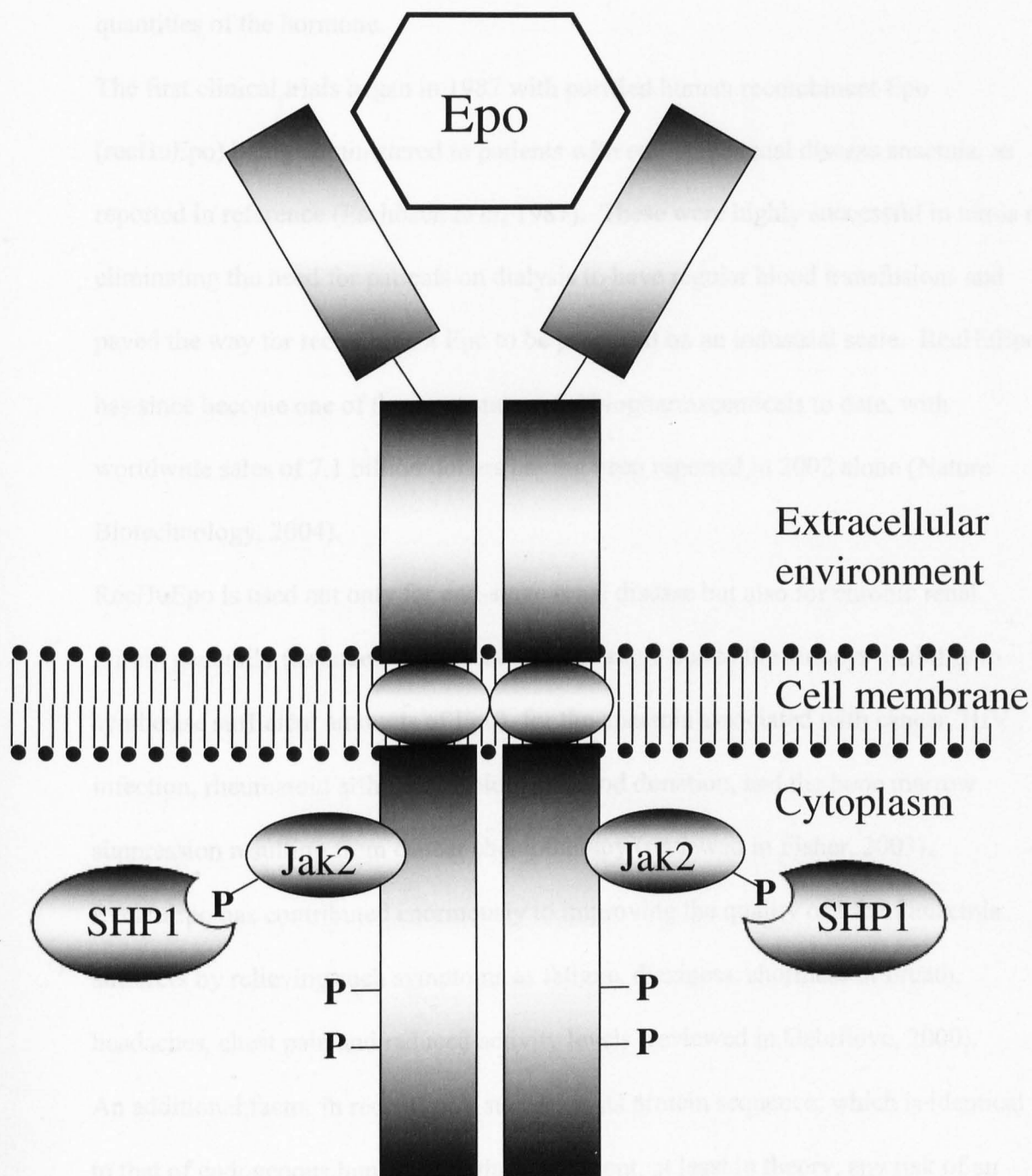
The cytoplasmic region of the Epo-R closest to the transmembrane domain is involved in the transduction of signals that lead to proliferation, whereas the C-terminal end has

a role in negatively regulating these signals (D'Andrea *et al*, 1991). The signalling pathways are strictly controlled and once activated are very promptly switched off. The dramatic decrease in response to Epo involves recruitment of phosphatases, such as SH2-containing protein tyrosine phosphatase 1 (SHP1), to the receptor and the Jak2 kinases. In addition, inhibitor proteins are produced and internalisation, ubiquitination and degradation of receptor-ligand complexes take place, thereby preventing the Epo-R from reappearing at the cell surface (Verdier *et al*, 2000; Walrafen *et al*, 2005). Signalling pathways then return to basal levels after 30-60 minutes (Verdier *et al*, 2000). Fig. 1.6.1. contains a schematic representation of the Epo-R and its associated signalling molecules.

### **1.7. The history of recombinant Epo and its medical applications**

Hormones (from the Greek word *hormon*, to "set in motion") are defined as substances produced in a given region of the body (usually an endocrine gland) which affect other parts of the body after being transported there by the blood. In 1906, Carnot and DeFlandre showed that injecting plasma from anaemic rabbits into normal rabbits prompted an increase in the number of circulating red blood cells, thus demonstrating that erythropoiesis was hormonally controlled (Carnot & DeFlandre, 1906). They gave the name "*hémopoiétine*" to the substance they considered responsible. Having found that this factor caused only the precursors of red blood cells to proliferate, Bondsdorff renamed it erythropoietin (Bondsdorff & Jalavisto E., 1948). In 1950, Reissman demonstrated that Epo production was stimulated by hypoxia (Reissman, 1950). Some seven years later, Jacobson *et al* (1957) showed by nephrectomy that Epo was produced in the kidney, but it was not until 1977 that Miyake and co-workers purified human Epo from 2,550 litres of urine from patients

Fig. 1.6.1. (Facing page) Structure of the human Epo receptor dimer and its associated signalling molecules. The human Epo receptor has a 225 aa extracellular domain, a 23 aa transmembrane domain and a 235 aa cytoplasmic domain. A 24 aa signal peptide is cleaved during post-translational processing. Epo binds the receptor's extracellular domain, inducing a conformational change that is transmitted to the cytosolic domain, leading to auto- or transphosphorylation of the Jak2 kinase. Jak2 then phosphorylates tyrosine residues of Epo-R, creating docking sites for the SH2 domains of several signal transduction proteins. Signalling is terminated principally by the protein tyrosine phosphatase SHP1 (modified from Ghaffari *et al*, 2003).





suffering from aplastic anaemia (Miyake *et al*, 1977). This made possible part-sequencing of the Epo protein, leading to the independent cloning of its gene by two groups in 1985 (Jacobs *et al* (1985) and Lin *et al* (1985)). The next step was expression of Epo in mammalian cells, thereby facilitating the production of greater quantities of the hormone.

The first clinical trials began in 1987 with purified human recombinant Epo (recHuEpo) being administered to patients with end-stage renal disease anaemia, as reported in reference (Eschbach *et al*, 1987). These were highly successful in terms of eliminating the need for patients on dialysis to have regular blood transfusions and paved the way for recombinant Epo to be produced on an industrial scale. RecHuEpo has since become one of the most successful biopharmaceuticals to date, with worldwide sales of 7.1 billion dollars having been reported in 2002 alone (Nature Biotechnology, 2004).

RecHuEpo is used not only for end-stage renal disease but also for chronic renal failure generally (as anaemia can result at any stage due to the kidneys' inability to synthesise sufficient amounts of Epo), for the anaemia associated with cancer, HIV infection, rheumatoid arthritis, autologous blood donation, and the bone marrow suppression resulting from cancer chemotherapy (reviewed in Fisher, 2003).

RecHuEpo has contributed enormously to improving the quality of life of anaemia sufferers by relieving such symptoms as fatigue, dizziness, shortness of breath, headaches, chest pain and reduced activity levels (reviewed in Gabrilove, 2000).

An additional factor in recHuEpo's success is its protein sequence, which is identical to that of endogenous human Epo, thus ruling out, at least in theory, any risk of an immune reaction. A number of cases have been reported, however, in which patients have developed antibodies against the administered recHuEpo. These antibodies

neutralise all Epo, including endogenous Epo, resulting in cessation of erythropoiesis, a condition called Pure Red Cell Aplasia (PRCA) (Rossert *et al*, 2004).

Conversely, the rise in the number of red blood cells brought about when excessive amounts of Epo are produced can cause the blood to become too viscous, thus increasing the risk of high blood pressure, thrombosis, strokes, heart failure, myocardial infarction and pulmonary embolism (Anderson & Scotti, 1972). This can occur naturally, as in the case of chronic mountain sickness, but it can also be caused by illegal doping – some athletes take recHuEpo as it can improve aerobic performance (Wagner, 1991).

### **1.8. Biologicals and batch testing**

Pharmaceuticals are, for regulatory purposes, regarded as falling into two groups: synthetic chemicals (small molecules, mostly) and biologicals (usually complex macromolecules). Biologicals have usually been defined as substances produced in biological systems which cannot be completely characterised by physico-chemical methods alone, and therefore also need to be tested by some form of bioassay. Their physico-chemical, biological and immunochemical properties must be fully characterised by the types of procedures set out in the International Conference on Harmonisation guidelines (1999). Unglycosylated proteins, such as insulin, pose less of a problem as they can be produced in large quantities in bacteria and with high levels of both purity and homogeneity. Bacteria are not, however, regarded as suitable for expressing glycosylated proteins as bacteria hardly glycosylate proteins at all and the glycan structures that are produced by prokaryotes differ too much from those of mammalian glycoproteins to be of any therapeutic value (Messner, 2004; Lindenthal &

Elsinghorst, 1999). Such glycoproteins need to be produced in mammalian cells, which are capable of complex glycosylation.

The polypeptide backbone of glycoproteins, including Epo, is invariant and strictly controlled by the encoding DNA sequence but there can be batch-to-batch variations in glycosylation. Fluctuations in the availability of glycosyltransferases lead to heterogeneity in the protein's oligosaccharide chains - the number of branches can vary as can the level of terminal sialylation. A range of possible factors have been suggested as being responsible, including host cell line, culture conditions, length of time in incubation, and method of purification (Goochee & Monica, 1990).

Glycosidases and proteases may also affect recombinant protein secreted into the culture medium (Le Floch *et al*, 2004). It is for this reason that batch testing is required.

There are two *in vivo* assays currently recognised by the European Pharmacopoeia (EP) for the batch testing of Epo (2005). Both are based on the premise that biologically active Epo stimulates proliferation of red blood cells.

### **1.9. The post-hypoxic polycythaemic mouse bioassay**

The post-hypoxic polycythaemic mouse bioassay involves placing mice in a hypobaric chamber with air at a pressure of half an atmosphere for 14 days. They become polycythaemic, eventually reaching a point where feedback inhibition takes place.

The consequential suppression of red blood cell production serves to create a low baseline from which to measure erythropoiesis stimulation (Cotes & Bangham, 1961).

Epo samples intended for testing are then administered on Day 14+1. Radiolabelled iron ( $^{59}\text{Fe}$ ) is injected on Day 14+3, becoming incorporated into freshly made

haemoglobin. The endpoint involves measuring the radioactivity in the red blood cells on Day 14+5.

Also available are the starved rat bioassay and the transfusion-induced polycythaemic mouse assay (the latter actually being more sensitive than the hypoxia-induced polycythaemic bioassay) but they are not used as they are more time, cost and labour intensive and place the animal in greater distress (Krumwieg *et al*, 1988).

### **1.10. The normocythaemic mouse bioassay**

Reticulocytes are immature anucleate red blood cells that still contain RNA and can be distinguished from mature anucleate RNA-free red blood cells. Kawamura *et al* (1991) developed a new *in vivo* bioassay based on measurement of immature reticulocytes in normal mice in response to subcutaneous Epo injections.

Normal animals are not responsive to small doses of exogenous Epo as the effect of such doses is masked by endogenous Epo. It is for this reason that the polycythaemic bioassay - which is more sensitive because of the suppression of endogenous Epo – is used in such instances.

The drawback of the polycythaemic mice bioassay is that it is time-consuming, cumbersome, expensive, and requires radioisotopes and elaborate animal preparations.

Mice suffer less in the normocythaemic mouse bioassay, as there is no need to pre-treat animals in a hypobaric chamber. It is also more precise than the polycythaemic mouse assay; even more so when, instead of one injection, three injections are carried out on successive days (Ramos *et al*, 2003).

Immature reticulocytes can be measured in a variety of ways: selective haemolysis of mature red blood cells leaving immature reticulocytes that can be counted with an automated cell counter, fluorescent flow cytometry (which makes use of thiazole

orange, a fluorescent dye that binds the RNA of immature red blood cells) or Cresyl Blue staining (a basic dye used for staining reticulocytes as it has a strong affinity for nucleic acid) (Kawamura *et al*, 1991; Hayakawa *et al*, 1992). The flow cytometry method combined with the multiple injection method has been found to give the greatest precision (Ramos *et al*, 2003). Although the normocythaemic assay is less invasive, it is only suitable when larger amounts of recHuEpo are available (as is likely in the case of recombinant Epo batch production).

Both assays involve comparing the Epo sample being tested with an international reference standard, the current one for Epo being the Second International Standard for Erythropoietin, NIBSC code: 88/574 (Storring & Gaines Das, 1992). By expressing potencies relative to a standard reference material, some of the major sources of variability between laboratories can be removed. Measuring the parallelism between the two curves can reveal whether the sample being tested is behaving in the same way as the reference standard. Both assays are specific for Epo and, in terms of sensitivity, the polycythaemic assay can detect 0.05IU of Epo, whereas the detection limit of the normocythaemic assay is 1IU (Hayakawa *et al*, 1992). Each assay uses 4-6 mice per treatment group.

There are also *in vitro* tests for Epo employing cells derived from sacrificed animals. While very sensitive as regards detecting concentrations, they cannot be used as a substitute for measuring *in vivo* potency. Examples of such tests include measuring the incorporation of  $^{59}\text{Fe}$  into haem of rat bone marrow cells (Goldwasser *et al*, 1975) or the proliferation and incorporation of  $^3\text{H}$ -Thymidine in spleen cells of phenylhydrazine-treated mice (Krystal, 1983). Treatment with phenylhydrazine is done because it results in fifty times more Epo-responsive cells being obtained from the spleen, an erythropoietic organ in mice (Rencricca *et al*, 1970).

### **1.11. Ethical issues**

As stated above, the general consensus on biopharmaceuticals has thus far been that their specific activity must be determined by some form of biological assay. *In vivo* assays are required in the case of certain glycoproteins, including Epo. Such tests can use large numbers of mice; no fewer than 42 are needed to test an unknown sample of Epo (six for each of three dilutions, the same again for the reference material, and six blanks). In 1959 William Russell and Rex Burch published "The Principles of Humane Experimental Technique", in which they outlined their 3Rs concept of refining, reducing or replacing animal experiments (Russell & Burch, 1959). They suggested that if animals had to be used in the course of experiments, every effort should be taken to refine those experiments so as to cause the animals the least possible pain and distress, and investigations should be aimed at reducing the number of animals needed while still maintaining statistical validity. The ideal course of action, however, would be to replace all *in vivo* tests with *in vitro* or physico-chemical alternatives. The European Union is committed to pursuing the same goal of improving the welfare of animals used in medical testing. According to directive 86/609/EEC of 24 November 1986 of the European Council, there is a requirement to support the development and validation of methods that have the potential to reduce, refine or replace the use of laboratory animals. Article 7.2 of the directive states that "an experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of the animal, is reasonably and practically available". The European Centre for the Validation of Alternative Methods (ECVAM) was set up in response to this directive. Article 7.3 of the directive states that "in a choice between experiments, those which use the minimum

number of animals . . . cause the least pain, suffering, distress, and lasting harm and which are most likely to provide satisfactory results shall be selected". One of their recommendations is therefore that the polycythaemic assay should, wherever possible, be replaced by the less invasive normocythaemic assay.

#### **1.11.1. The suitability of replacing *in vitro* with *in vivo* assays**

Small unglycosylated peptide hormones, such as oxytocin and calcitonin (9 and 32 amino acids long respectively), are examples of suitable for being quantified by High Performance Liquid Chromatography (HPLC), a physico-chemical method, and not by an animal test (Miethe, 2002). Immortalised cell line bioassays can be used to assess the potency of non-glycosylated proteins (or proteins for which glycosylation is not crucial to activity) such as interferons (Meager, 2002), colony-stimulating factors (Metcalf *et al*, 1994), and interleukins (Mire-Sluis & Thorpe, 1998). Glycoproteins such as Epo pose more of a problem for researchers seeking to reduce the amount of testing done on animals. This is because the *in vivo* activity of Epo is dependent on its glycosylation state, most critically on its sugars being capped with sialic acid (also known as N-acetylneuraminic acid), a factor that is more or less irrelevant to its effect on receptor binding and *in vitro* activity. Glycosylation is not a tightly regulated process and results in Epo being made up of a mixture of glycoforms. Assigning a potency to such a heterogeneous pool of proteins using a technique such as HPLC is no simple matter; determining how much of a substance is present in a preparation in terms of mass or number of moles does not necessarily reveal how active that substance is. The data obtained by immunological and cell-based assays for Epo do not correlate with those obtained by *in vivo* bioassays. Proliferation of Epo-dependent cells depends solely on the binding of Epo to the erythropoietin receptor and on

successful transmission of a proliferative/anti-apoptotic signal from the exterior of the cell to its interior. *In vivo* activity, on the other hand, is dependent not only on these same events taking place in and on target cells but also on the hormone's pharmacokinetics, including how it is removed by the body's clearance mechanisms.

## **1.12. Clearance mechanisms**

The faster a hormone is cleared from the body, the less time it will have to elicit a reaction from its target cells. There are a number of mechanisms by which a hormone is cleared from the body, and they will all have a bearing on its effective potency.

### **1.12.1. Hepatic clearance of desialylated glycoproteins**

In their seminal paper, Morell *et al* (1968), reported the finding that radioactively labelled neuraminidase-treated ferroxidase, a copper transport glycoprotein found in the blood, cleared more rapidly from the circulation of rabbits than did the unmodified form of the glycoprotein. Radioactivity accumulated in parenchymal cells of the liver. Replacing the missing sialic acids of desialylated ferroxidase or treating it with galactose oxidase or  $\beta$ -galactosidase, and thereby eliminating the presence of exposed terminal galactose residues, resulted in a return to the same pharmacokinetics as those witnessed for intact ferroxidase. These experiments were repeated by Spivak and Hogans, this time using Epo (1989), and showed that oxidation of asialo-Epo's galactose residues again restored clearance rates to those of fully sialylated Epo despite the *in vitro* activity being abolished. Co-injection of excess asialo-alpha-1-acid glycoprotein reduced the accumulation of asialo-Epo in the liver, something not achieved with sialylated alpha-1-acid glycoprotein. Goldwasser *et al* (1974) were the first to show that desialylation of Epo resulted in a loss of *in vivo* biological activity



while *in vitro* activity remained intact and similar effects have been reported in numerous papers (Dordal *et al*, 1985; Wasley *et al*, 1991; Tsuda *et al*, 1990).

Experiments of this kind have been carried out with a wide variety of glycoproteins in addition to Epo and ferroxidase: alpha-1-acid glycoprotein, fetuin, haptoglobin,  $\alpha_2$ -macroglobulin, chorionic gonadotropin and follicle-stimulating hormone, demonstrating the apparent universality of this phenomenon (Morell *et al*, 1971).

Asialo-Epo clears from the circulation in six minutes whereas Epo produced in cells that can transfer only truncated glycan structures takes 90 minutes, showing that the exposed terminal galactose is the crucial determining factor in clearance (Wasley *et al*, 1991).

A galactose-specific liver receptor responsible for binding the penultimate galactose residues left exposed through removal of terminal sialic acids by neuraminidase was postulated to exist. A candidate for such a receptor was isolated and characterised by Pricer *et al* (1974) and named the asialoglycoprotein receptor (ASGP-R).

The role the liver's ASGP-R plays in clearing such experimentally desialylated glycoproteins is thus a widely reported and accepted phenomenon. This does not necessarily reflect what takes place in the body in actual physiological terms, however, or explain the true role of the ASGP-R.

Tozawa *et al* (2001) speculate on how the ASGP-R may have evolved for a completely different reason - to protect mammals from viruses and/or bacteria. Some pathogens may use neuraminidase to invade their host and so galactose residues would be a sign of such an infection. The authors note that people with liver disease frequently develop serious infections such as spontaneous bacterial peritonitis and sepsis. Weigel (1994) expounds on the view that glycoproteins with exposed galactose residues could be harmful and need to be cleared but admits there is no

explicit evidence for this. The fact that birds and reptiles do not possess an ASGP-R and have a higher concentration of circulating asialo-glycoprotein than do mammals (Regoecki *et al*, 1975) may or may not be of particular significance. The most widely held view is that glycoproteins are cleared by the ASGP-R after they have served a particular physiological function (Ashwell & Harford, 1982); a potential scenario being that a sialidase acts on circulating glycoproteins and that the chance of desialylation occurring increases with time. The theory relies on there being a circulating form of neuraminidase and one of these has already been reported (Corfield *et al*, 1982). There is also a report of low levels of a neuraminidase in Cohn Fraction VI from mammalian plasma (Warren & Spearing 1960).

Appealing though this theory may be, it is unsupported by direct evidence (Drickamer, 1988). There is, in fact, evidence to the contrary. Lefort *et al* (1984) injected intact human chorionic gonadotropin into rats and found that asialo-fetuin does not compete with its clearance nor is there accumulation of desialylated human chorionic gonadotropin in the circulation; evidence that any clearance mechanisms probably do not involve glycoproteins becoming desialylated (by a sialidase, for example), thereby mediating uptake via the ASGP-R. Wong *et al* (1974) assert that neither does transferrin undergo *in vivo* desialylation.

Direct correlation has been claimed, however, between extent of liver damage or the size of a liver cell carcinoma and the amount of circulating asialoglycoproteins (Marshall *et al*, 1974; Sawamura *et al*, 1984). There is speculation that this could be due to the liver no longer being able to clear asialoglycoproteins from the circulation or, alternatively, that the liver could be incorrectly modifying proteins post-translationally. Coincidentally or otherwise, analysis of the hepatocytes of patients with cirrhotic liver disease revealed the ASGP-R (usually all but restricted to

the sinusoidal surface) to be redistributed around the cell (Burgess *et al*, 1992). By contrast, Jensen *et al* (1995) found Epo pharmacokinetics to be the same in advanced liver cirrhosis patients as in healthy volunteers.

Tozawa *et al* (2001) reported on a knockout mouse lacking the ASGP-R gene. While in mice of this type artificially desialylated proteins (asialo-orosomucoid and asialo-fetuin) were not cleared at as fast a rate, plasma glycoproteins levels - as detected by blotting with lectins specific for galactose and sialic acid - did not increase. Deal *et al* found that the liver of ASGP-R-deficient mice still takes up radiolabelled galactosylated bovine serum albumin (BSA) but that more radioactivity accumulates in endothelial liver cells compared with wild-type mice – possible evidence for a compensatory mechanism or overlap of function (Deal *et al*, 1998). There is a macrophage galactose/N-acetylgalactosamine lectin that could be playing a part in compensating for the missing ASGP-R (Ii *et al*, 1990; Suzuki *et al*, 1996).

### **1.12.2. Renal clearance**

It has been reported that urinary Epo contains more acidic isoforms than does serum Epo, leading to speculation that urinary Epo is Epo that appears in the urine because it is cleared less rapidly by the liver (Storring & Gaines Das, 1992; Tam *et al*, 1991; Wide & Bengtsson, 1990). Fukuda *et al* (1989) injected radiolabelled desialylated Epo into rats and measured the radioactivity of four organs after 30 minutes. The liver accumulated the most radioactivity (85% of total radioactivity), followed by the kidneys (9%), lungs (4%) and spleen (2%). Intact, fully sialylated Epo was distributed as follows: liver 64%, kidney 24%, lung 9% and spleen 3%. Once again, accumulation took place mainly, but to a lesser extent, in the liver, and the kidneys became a site of further accumulation. This is additional support for the hypothesis

that if Epo does not lose sialic acids in time to engender clearance by the liver, then more acidic forms of Epo are cleared by the kidneys.

Interestingly, a form of Epo called Epo-bi has been studied that is rich in bi-antennary sugars and accumulates to a greater extent in the kidneys compared with tetra-antennary Epo, making for faster clearance from the circulation (Takeuchi *et al*, 1989). The faster rate of clearance is not observed in rats whose kidneys have been removed (Misaizu *et al*, 1995). The presence of bi-antennary sugars on prolactin makes for a swifter clearance by the kidneys, whereas if there are no sugars it is cleared more slowly by the liver (Hoffmann *et al*, 1993). This would imply that a smaller size due to bi-antennary sugars cannot be the sole reason for the clearance of bi-antennary glycosylated proteins by the kidneys as deglycosylated protein is smaller. This suggests that the kidney has a role in clearing Epo containing incomplete carbohydrate branching.

### **1.12.3. Receptor-mediated clearance**

Studies of Epo clearance in sheep showed no difference before and after hepatectomy or nephrectomy, leading researchers to consider the possibility that receptor-mediated endocytosis is the most prevalent mechanism by which Epo is cleared (Widness *et al*, 1996). However, the glycosylation of the Epo used in this study was not characterised and if the Epo were well sialylated, a hepatectomy might not be expected to affect clearance.

Support for receptor-mediated endocytosis is provided by the observation that Epo clearance is reduced following bone marrow ablation by busulfan (Chapel *et al*, 2001), suggesting that Epo is cleared mainly by Epo receptor-displaying erythroid

progenitors within the bone marrow. In a contradictory study (Piroso *et al*, 1991), the half-life of Epo was unaffected by suppression or stimulation of bone marrow. Studies of asialo-transferrin have revealed some interesting, if not completely resolved, results (Regoezi *et al*, 1980). The glycoprotein transferrin exists as two main isoforms that vary in terms of their sugar branching. It either possesses two bi-antennary glycans or one bi-antennary and one tri-antennary glycan. The 2x bi-antennary form has lower affinity for the liver lectin but is cleared earlier in rabbits. The authors found that it is cleared in the bone marrow and that the process is galactose-specific. Initially the 1x bi-antennary 1x tri-antennary is cleared at a faster rate but then the 2x bi-antennary overtakes and is depleted sooner, indicating an additional site of catabolism. Leucocytes have a preference for bi-antennary glycans whereas the liver has preference for tetra-antennary (Bezouska *et al*, 1985). It is not simply the case that the removal of sugars may make it easier to bind its specific receptor as rabbit asialo-transferrin with just one bi-antennary type glycosylation site binds neither liver nor bone marrow.

#### **1.12.4. Additional roles of sugars**

Glycosylation plays a number of protective roles in addition to determining the rate of clearance by the liver and kidneys: it facilitates protein secretion, aids protein folding, reduces susceptibility to thermal inactivation (Tsuda *et al*, 1990) and confers protection against degradation by proteases. Asialo-epo has been reported as being more sensitive to heat denaturation and trypsin action (Goldwasser *et al*, 1974; Briggs *et al*, 1974) whereas Carter *et al* (2004) observed that deglycosylated granulocyte-colony stimulating factor was rendered biologically inactive following incubation in serum, presumably owing to the action of proteases. Lactoferrin was

also reported as exhibiting the same property when incubated with trypsin (van Berkel *et al*, 1995; van Veen *et al*, 2004). By contrast, the glycosylated forms of these cytokines are protected from such degradation. Sugars can also potentially influence the binding affinity of a ligand for its receptor and activation of intracellular signal transduction (although Epo glycosylation does not affect the latter).

### **1.13. Structure of the ASGP-R**

The role the liver's galactose-binding receptor plays in clearing asialoglycoproteins is one of the most widely studied clearance mechanisms. The receptor itself is very well characterised and was used in expression studies described in Chapter 4. The ASGP-R is predominantly expressed on the sinusoidal surface of the parenchymal cells of the liver (Wall & Hubbard, 1981). It is an oligomer consisting of two subunits, H1 and H2, coded for by two different but highly homologous genes, sharing 55% sequence identity (Bischoff & Lodish, 1987; Bischoff *et al*, 1988; Spiess & Lodish, 1985). The mature and fully glycosylated forms of the H1 and H2 subunits are ~46 and ~50 kDa respectively (Bischoff & Lodish, 1987; Baenziger & Maynard, 1980). The ASGP-R is a single-spanning membrane protein with an extracellular calcium-dependent galactose/N-acetylgalactosamine-binding domain and therefore belongs to the  $\text{Ca}^{2+}$ -dependent (C-type) family of animal lectins (carbohydrate-binding proteins). It is a type II membrane receptor, characterised by a cytosolic amino-terminal domain (~40 amino acids), a single-spanning transmembrane domain (~20 amino acids), a stalk segment (~80 amino acids), and an extracellular carboxy-terminal carbohydrate recognition domain (~150 amino acids) (Bider *et al*, 1996). This is in contrast to a Type I receptor, such as the Epo receptor,

which has the reverse orientation in the membrane (see Fig. 1.13.1. for a schematic diagram of the ASGP-R).

Asialoglycoprotein binds to the ASGP-R on the surface of the cell at neutral pH. The asialoglycoprotein ligand becomes dissociated in the low-pH environment of the endosome, followed by degradation in the lysosome while the receptor is recycled back to the cell membrane (Geffen *et al*, 1989). The H1 subunit can be stably expressed by itself whereas in the absence of H1 most of H2 is unstable and is degraded in the endoplasmic reticulum (ER) (Shia & Lodish, 1989; Amara *et al*, 1989). Additional evidence for this was provided by Tozawa *et al* (2001), who reported that H2 was undetectable in a knock-out mouse lacking the gene for the H1 analogue. H1 is still expressed if H2 has been knocked out but in reduced amounts (Ishibashi *et al*, 1994).

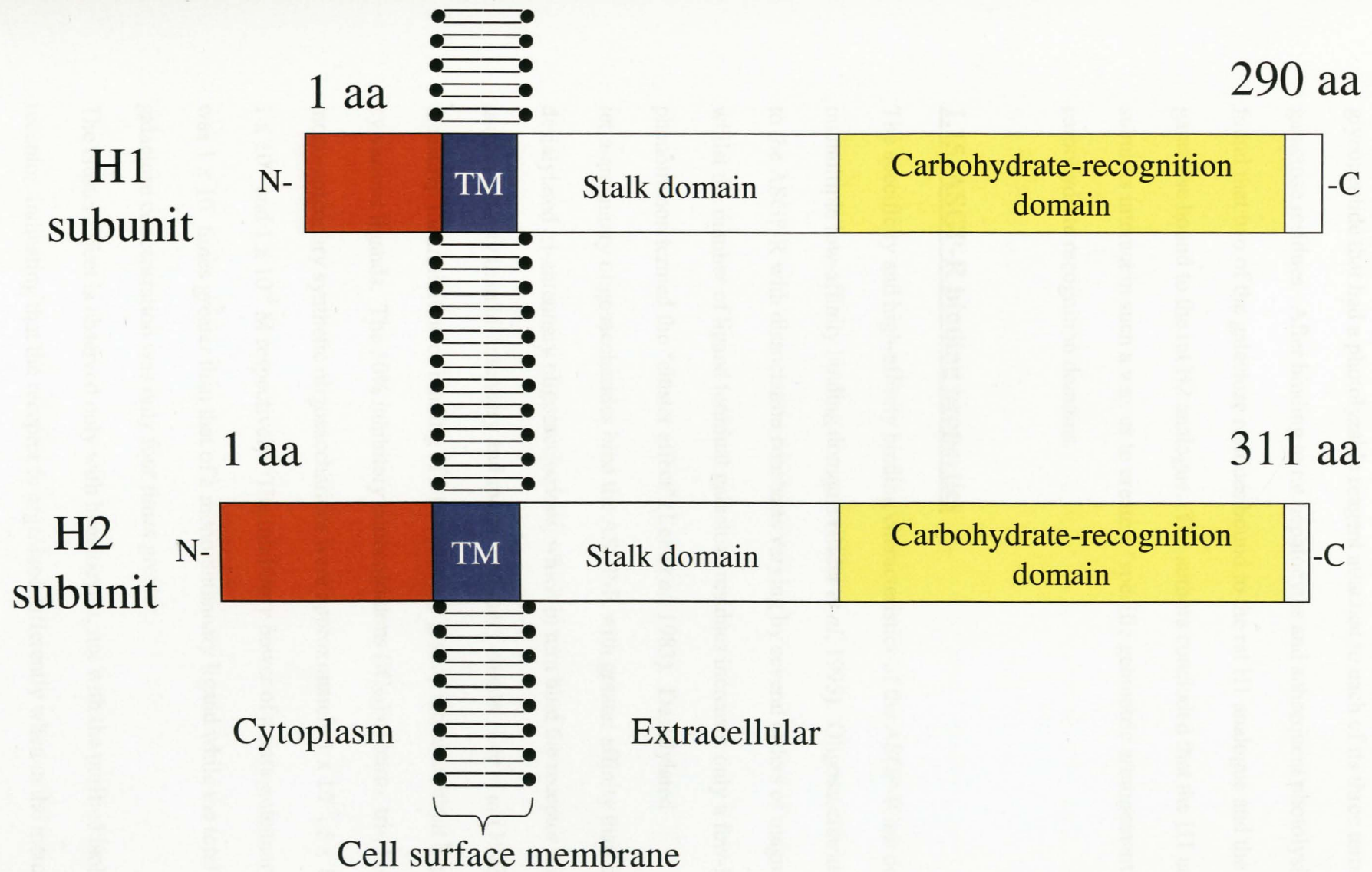
For high binding affinity, both subunits need to be expressed. H1 expressed on its own forms homo-oligomers that can bind asialo- $\alpha$ -1-acid glycoprotein, but with low affinity and only if overexpressed (Bider *et al*, 1995). It can be speculated that the presence of H2 enhances the spatial organisation of the carbohydrate recognition domains, thereby facilitating optimised binding affinity.

#### **1.14. H1 to H2 binding ratios**

The H1 and H2 subunits are reported to oligomerise and indeed, their stalk regions contain heptad repeats that are characteristic of proteins which oligomerise via  $\alpha$ -helical coiled coils (Bider *et al*, 1996). Evidence differs, however, regarding the H1:H2 binding ratios with Lederkremer and Lodish (1991) putting it at 3:1 or 4:1, whereas according to Bider *et al* (1996) it is 2:2 if the subunits are at the concentration found in HepG2 cells (a human liver cell line).

Fig. 1.13.1. (Facing page) Schematic representation of the two subunits of the asialoglycoprotein receptor (ASGP-R). Note the type II receptor orientation, in which the N-termini are positioned inside the cell (derived from information in the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics).





TM= transmembrane domain and signal anchor

In order to investigate this further, Rice *et al* (1990) designed a triantennary glycopeptide that had a photolyzable reagent attached to each of its three terminal galactose residues. After binding to rat hepatocytes and subsequent photolysis, it was found that two of the galactose residues bound to the rat H1 analogue and the third galactose bound to the rat H2 analogue. The authors concluded that the H1 and H2 subunits interact in such a way as to create a "specific geometric arrangement" of the carbohydrate recognition domains.

### **1.15. ASGP-R binding properties**

The specificity and high-affinity binding characteristics of the ASGP-R are dependent on multiple low-affinity binding domains (Bider *et al*, 1995). Oligosaccharides bind to the ASGP-R with dissociation constants varying by several orders of magnitude whilst the number of ligand terminal galactose residues increases only a few-fold – a phenomenon termed the "cluster effect" (Lee *et al*, 1983). Desialylated tetra-antennary oligosaccharides bind the ASGP-R with greater affinity than do desialylated tri-antennary oligosaccharides, which in turn bind the receptor with even greater affinity than bi-antennary and mono-antennary chains. Lee *et al* (1983) measured the inhibition of binding of a triantennary glycopeptide to rabbit hepatocytes by various ligands. The 50% inhibitory concentrations ( $IC_{50}$ ) of tetra-, tri-, bi-, and mono-antennary synthetic oligosaccharides were approximately  $1 \times 10^{-9}$ ,  $5 \times 10^{-9}$ ,  $1 \times 10^{-6}$  and  $1 \times 10^{-3}$  M respectively. The inhibitory power of a tetra-antennary ligand was  $1 \times 10^6$  times greater than that of a mono-antennary ligand while the total galactose concentration was only four times greater.

The cluster effect is observed only with hepatocytes, not with the purified isolated receptor, indicating that the receptor is organized differently when in the membrane

(Connolly *et al*, 1982; Baenziger & Fiete, 1980). In contrast to the membrane-bound receptor, the isolated human receptor is inhibited by asialo-triantennary and asialo-biantennary oligosaccharides with  $IC_{50}$  values of  $1.3 \times 10^{-6}$  and  $3.9 \times 10^{-5}$  M respectively (Baenziger & Maynard, 1980).

The above-mentioned Rice *et al* paper (1990) supports this hypothetical difference in spatial arrangements in the membrane because, if soluble rat liver receptor is used, the glycopeptide does not bind to H2. In conclusion, H1 and H2 need to associate to have full binding properties.

The binding of a galactose residue to an individual receptor subunit is of low affinity and therefore high affinity binding is the result of at least three galactose residues interacting simultaneously with three subunits, forming an oligomeric complex. If it is assumed that the ASGP-R binds three galactose residues of an oligosaccharide then this would explain why the difference in inhibitory concentration between tetra-antennary and tri-antennary is not as great as that between all the other species of oligosaccharide.

In addition to the galactose/N-acetylgalactosamine-binding ASGP-R, there is a range of animal lectins with different specificities (Drickamer & Taylor, 1993). All C-type lectins have a similar structure but can vary in their sugar binding characteristics (e.g. mannose/glucose/N-acetylglucosamine/fucose versus galactose/N-acetylgalactosamine); these being the result of just a few changes in amino acid sequence (Iobst & Drickamer, 1994).

Many studies have been carried out with the aim of exploiting the ASGP-R and the fact that it is, for the most part, expressed exclusively by the liver. Drugs and genes can be specifically targeted to the liver by conjugation with high affinity ligands for the ASGP-R (see (Wu *et al*, 2002) for review). This research again confirmed that the

two most important factors that govern binding to the ASGP-R are the branching pattern of the oligosaccharide and the distance between its terminal galactose residues. The ASGP-R is also suspected of being used as the entry site for Hepatitis A virus (Dotzauer *et al*, 2000), Hepatitis B virus (Treichel *et al*, 1997), Hepatitis C virus (Saunier *et al*, 2003) and the Marburg virus (Becker *et al*, 1995). The Marburg virus has terminal galactose residues on its surface glycoproteins and it is therefore suggested that it uses the ASGP-R as a point of entry into hepatocytes. Asialo-fetuin inhibits the attachment. Hepatitis B virus is also inhibited by asialo-fetuin (Treichel *et al*, 1994).

### **1.16. Objectives**

Replacement of the Epo *in vivo* assays with an *in vitro* method would result in a reduction in the number of animals sacrificed and a reduction in costs incurred (testing one Epo sample against a reference standard in the normocythaemic assay costing approximately £1510), and could potentially improve the level of precision. Current *in vitro* assays for Epo are all but unaffected by their level of glycosylation, which is why correlation is not obtained between bioactivities obtained by *in vivo* methods and by immuno- or cell-based assays (Yamaguchi *et al*, 1991). One aim of this thesis was to test whether it was feasible to manipulate *in vitro* assays in such a way that rendered them sensitive to the factors that affect *in vivo* assays. To what extent would correlation be achieved between potencies obtained by such assays and by the mouse bioassays? The most important property of such an assay would be the ability to differentiate between sialylated Epo and desialylated Epo. Three approaches to this problem were pursued although all shared the common rationale of incorporating a mechanism for mimicking the action of the liver.

An additional objective was to determine how effective such a strategy might be in comparison with other currently available biological and physico-chemical techniques when used on three different Epo sample sets, each one presenting a different pharmaceutical situation – a neuraminidase-treated sample set, a set whose samples differed in terms of culture conditions and purification procedures, and a single batch of pharmaceutical-grade Epo separated into fractions by anion-exchange chromatography. An assessment could then be made of the potential for reducing the requirement for animal testing.

## **Chapter 2. Materials and Methods**

Water was either MilliQ grade (equivalent to double distilled H<sub>2</sub>O) or, for molecular biology applications, "sterile", i.e. purified using the Barnstead Thermolyne NANOpure Diamond Life Science UV/UF ultrapure system and autoclaved (nuclease-, DNA- and pyrogen-free).

All reagents were obtained from Sigma unless otherwise stated.

### **2.1. Molecular Biology Techniques**

#### **2.1.1. Messenger RNA purification**

Messenger RNA (mRNA) was purified from mammalian cells using the QuickPrep mRNA Purification Kit (Amersham Biosciences, UK), following the manufacturer's protocol. Briefly, after guanidinium thiocyanate inactivation of endogenous RNases in the cell extract, mRNA was isolated by exploiting the fact that its 3'-terminal poly(A) tracts bind to oligo(dT)-cellulose beads. A series of high- and low- salt buffer washes was then followed by a final elution step.

Total RNA was isolated from plant seeds using the plant RNeasy Plant mini-kit (Qiagen). The seeds were homogenised under liquid nitrogen in a pestle and mortar and RNA was extracted according to the manufacturer's instructions. The kit involves a guanidine-isothiocyanate lysis step, followed by passage through a QIAshredder column which homogenises and filters viscous plant lysates, and finally silica-gel-membrane purification.

### **2.1.2. First-strand cDNA synthesis**

First-strand cDNA was produced from mRNA using reverse transcriptase reagents provided by Amersham Biosciences.

*A typical reaction:*

Reagent	Volume ( $\mu$ l)
Bulk First-Strand Reaction Mix (containing Murine Reverse Transcriptase [5 units/ $\mu$ l], Tris buffer at pH 8.3, KCl, MgCl <sub>2</sub> , BSA, dNTPs)	11
200 mM dithiothreitol (DTT)	1
Primers (pd[N] <sub>6</sub> )	1
RNA template (prepared as described in Section 2.1.1.)	20

Prior to adding all reagents to a sterile thin-walled 500  $\mu$ l PCR tube, the RNA was heated at 65°C for 10 minutes, then chilled on ice in order to remove RNA secondary structure. Following brief mixing and centrifugation the solution was incubated at 37°C for one hour.

The resulting cDNA product was stored at -40°C.

### **2.1.3. Polymerase chain reaction**

#### **2.1.3.1. Design of primers**

Primers were designed according to the relevant sequences in the GenEMBL database. The 10 bases preceding the start codon (the Kozak sequence) were amplified in addition to the open reading frames because the presence of this sequence has been shown to optimise translation (Kozak, 1987). In some cases primers also contained a

restriction site to facilitate cloning, plus a few extra bases should the ends be degraded during the PCR process. Where appropriate, the 3' primer contained a stop codon.

The primers used in plasmid construction were synthesised by Eurogentec (Southampton, UK) or VH BIO (Newcastle, UK).

### 2.1.3.2. PCR mix

The Expand High Fidelity PCR System (Roche, UK) was the principal method used for PCR-amplification of the DNA. The kit contains an enzyme mix combining the high fidelity of the proof-reading *Tgo* DNA polymerase and the high yield of *Taq* DNA polymerase.

*For a 50 µl typical reaction:*

Reagent	Volume (µl)	Final concentration
10 x Expand High Fidelity buffer with 15 mM MgCl <sub>2</sub>	5	1 x
dNTPs (1.25 mM each)	8	0.2 mM
Forward primer (20 µM)	5	2 µM
Reverse primer (20 µM)	5	2 µM
Sterile water	25	-
Expand High Fidelity enzyme mix (3.5 U/µl)	1	0.07 U/µl
Template (1 <sup>st</sup> strand cDNA or plasmid diluted 1/1000)	1	-

All reagents except the template were placed in a sterile thin-walled 500 µl PCR tube (Scientific Specialties, Inc, USA) and heated to 94°C in the Techne Genius PCR machine/thermal cycler (Techne Ltd, Cambridge, UK) for 1 minute to allow them to heat up. The template was then added and the PCR cycle initiated.



*A typical PCR cycle:*

Step	Temperature	Time	Number of cycles
Initial denaturation	94°C	1 min	1
Denaturation	94°C	30 secs	30
Annealing	55°C	30 secs	
Elongation	72°C	1 min per kbp	

On completion of the cycles the temperature was held at 72°C for 10 minutes, during which time an increased number of 3'-A overhangs were added on to aid in TA-cloning.

*Pfu* DNA polymerase (Stratagene, UK) was occasionally used in PCR-amplification because, although it is less reliable in terms of yield, it has the potential for greater accuracy. Since *Pfu* DNA polymerase produces blunt-ended PCR products, the latter have to undergo an A-tailing procedure.

*The A-tailing reaction (10 µl):*

Reagent	Amount
PCR fragment (purified as described in Section 2.1.11. in order to remove proof-reading enzyme)	6.4 µl
10 x Expand High Fidelity buffer with 15 mM MgCl <sub>2</sub>	1 µl
dATP (1.25 mM)	1.6 µl
<i>Taq</i> DNA polymerase (5 units/µl, Promega, UK)	1

The reaction was then incubated at 70°C for 30 minutes.

#### **2.1.4. TA cloning**

PCR products (from Section 2.1.3.) were cloned into the pGEM-T “T vector”

(Promega, UK) using T4 DNA ligase and transformed into *Escherichia coli* (*E. coli*)

JM109 cells (see Section 2.1.5. below). See Fig. 2.1.1. for a diagram of pGEM-T.

*pGEM-T ligation reaction mix (10 µl):*

Reagent	Volume
2x Rapid Ligation Buffer (containing 60 mM Tris-HCl [pH 7.8], 20 mM MgCl <sub>2</sub> , 20 mM DTT, 2 mM ATP and 10% polyethylene glycol)	5 µl
pGEM-T Vector (50 ng)	1 µl
PCR product from Section 2.1.3.	2 µl
T4 DNA Ligase (3 units/µl)	1 µl
Sterile water	1 µl

#### **2.1.5. Bacterial transformation**

##### **Competent cells**

Transformations were carried out using *E. coli* JM109 (Promega, UK) and SCS110 (Stratagene, UK) competent cells.

##### **Media**

See Appendix for Luria-Bertani (LB) medium, LBamp and LBamp/IPTG/X-Gal plate recipes.

##### **Transformation of bacteria**

*E. coli* JM109 competent bacteria (Promega, UK) initially underwent gentle thawing on ice. For transformation, 2 µl of ligation product was added to 100 µl of the

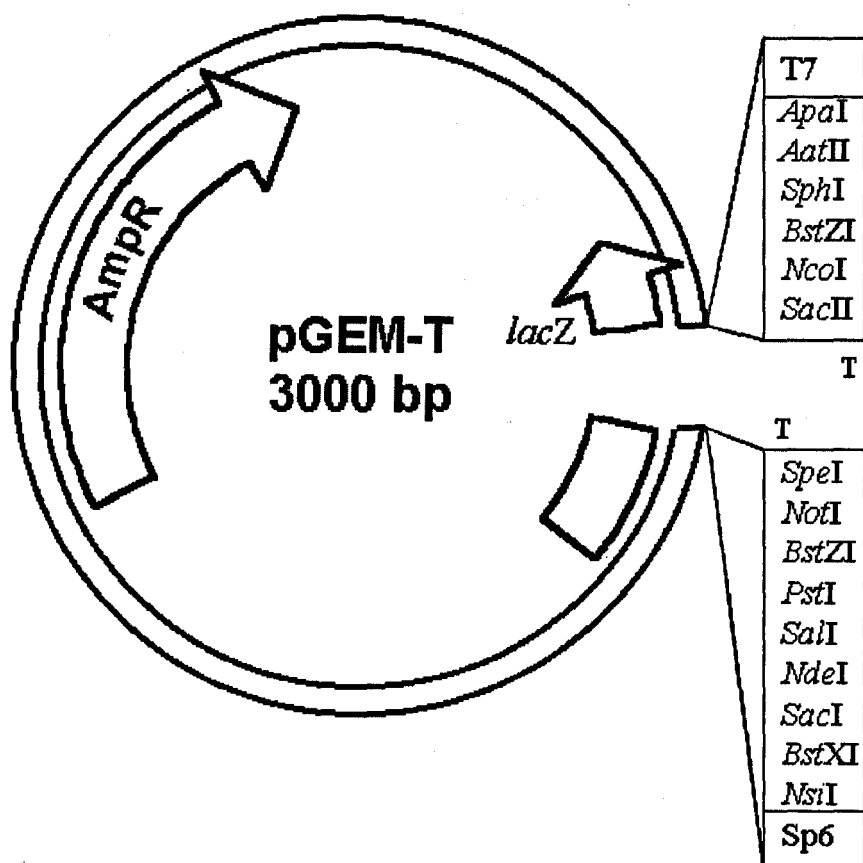


Fig. 2.1.1. Diagram of the pGEM-T vector (Promega). Note the overhanging Ts used in TA cloning (Section 2.1.6.), the *lacZ* gene used in blue/white screening, and the multiple cloning site (useful for fusion protein construction (Chapter 5)).

competent cells in a 14 ml Falcon tube (BD Biosciences Catalog #352059) and chilled on ice for 30 minutes. The cells were then heat-shocked at 42°C for 45 seconds and placed on ice for a further two minutes. Sterile LB medium (500 µl) was added to the cells, which were incubated at 37°C for 1 hour with shaking at 200 rpm. Then, 100 µl of the transformation reaction was added to an LBamp plate or an LBamp plates with IPTG/X-Gal and incubated overnight at 37°C.

*E. coli* SCS 110 bacteria, which are deficient in two methylases (Dam and Dcm) and therefore incapable of methylating plasmid DNA, were used when the restriction enzyme *Cla*I, the site of which is methylation-sensitive when preceded by a G or proceeded by a C base, was needed for subsequent subcloning steps. This strain was transformed using the above method, but with the following two modifications: to improve transformation efficiency, SCS110 (100 µl) were incubated for ten minutes with 1.7 µl of β-mercaptoethanol prior to addition of the DNA; and LB medium was replaced by SOC medium (see Appendix for medium recipe).

#### **2.1.6. Blue/white screening**

The pGEM-T vector (first referred to in Section 2.1.4.) allows blue/white screening of recombinants as it contains the Lac Z gene, which codes for β-galactosidase. This enzyme hydrolyses 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to produce a blue colony. Isopropyl β-D-thiogalactoside (IPTG) acts as an inducer of the gene. Introduction of a coding sequence into the Lac Z gene disrupts its expression, resulting in the production of white recombinant colonies. Cultures of cells picked from the white colonies were grown up and their plasmid DNA extracted (see description in Section 2.1.7. below).

### **2.1.7. Preparation of DNA from transformed *E. coli***

A single colony containing plasmid DNA was touched with a pipette tip which was then dipped into a Falcon polypropylene round-bottom tube (BD Biosciences Catalog #352059) containing 2 ml LBamp. The cultures were incubated overnight at 37°C with shaking at 225 rpm, whereupon 1.5 ml of the culture was transferred to a microfuge tube and the contents centrifuged at 13,000 rpm for 5 minutes to pellet cells. The supernatant was aspirated and the pellet resuspended in 100 µl of solution P1 (see Appendix for solution recipes) by vortexing.

Solution P2 (200 µl) was added to the tube and the contents were mixed by inversion, before incubating at room temperature for 5 minutes. This solution causes lysis of cells and the separation of genomic DNA from plasmid DNA.

Chilled solution P3 (150 µl) was added to neutralise the contents and mixed by inversion.

The tube was left on ice for 5 minutes before centrifuging for 5 minutes at 13,000 rpm to pellet cellular debris. The supernatant was transferred to a new tube, an equal volume of phenol:chloroform:isoamyl alcohol 25:24:1 mixture (Sigma, UK) was added and the tube was then vortexed. After centrifugation (at 13,000 rpm for 5 minutes) the bottom organic layer was removed by pipetting and discarded. Twice the volume (0.8 ml) of 100% ethanol was added to the tube and the contents were mixed by inversion. After centrifugation (at 13,000 rpm for 10 minutes) the supernatant was aspirated leaving the DNA pellet. The pellet was washed in 80% ethanol to remove any salts left over from the buffer. The DNA was air-dried for 10 minutes and resuspended in 50 µl RNase-containing water (20 µg/ml) and stored at -40°C. The DNA was analysed for the presence of a DNA insert by digestion using the appropriate restriction endonuclease (Section 2.1.8.).

Approximately 200  $\mu\text{l}$  of the remaining overnight culture was added to an equal volume of 30% glycerol in LB and stored at  $-80^{\circ}\text{C}$ .

### **2.1.8. Restriction endonuclease digestion plasmid DNA**

Endonuclease reactions were performed using Amersham Biosciences, Promega or New England Biosciences (NEB) products.

*A typical restriction enzyme digestion was as follows (10  $\mu\text{l}$ ):*

Reagent	Volume
Plasmid	2 $\mu\text{l}$
10x One-Phor-All (OPA) buffer (Amersham Biosciences, UK) or the appropriate 10x NEB buffer	1 $\mu\text{l}$
Restriction enzyme A ( $\sim 5$ units/ $\mu\text{l}$ )	0.25 $\mu\text{l}$
Restriction enzyme B ( $\sim 5$ units/ $\mu\text{l}$ )	0.25 $\mu\text{l}$
Sterile water	6.5 $\mu\text{l}$

The digests were incubated for two hours at the optimum temperature for enzyme action (usually  $37^{\circ}\text{C}$ ). Then, 2  $\mu\text{l}$  6x DNA gel loading buffer was added to the digestion mixture and the DNA was resolved on a 1% agarose gel (1.5% when analysing fragments less than 500 bp) to confirm the digestion.

### **2.1.9. Electrophoresis of DNA**

#### *1% agarose gels*

A quantity (0.5 g) of electrophoresis-grade agarose (Amersham Biosciences, UK) was added to 50 ml of 1x TBE buffer (Sigma, UK), and the mixture heated in the microwave for about two minutes until the agarose had dissolved. The solution was cooled to about  $50^{\circ}\text{C}$  and 25  $\mu\text{l}$  of 1 mg/ml Ethidium Bromide (final concentration of 0.5  $\mu\text{g/ml}$ ) was added. The mixture was poured into a gel tray and allowed to set.

### *Gel electrophoresis*

Electrophoresis was performed using a horizontal electrophoresis system (BDH, UK). Samples (10 µl) were loaded onto the gel in an electrophoresis tank filled with 1x TBE running buffer and run against a 0.05-10 kb DNA ladder (Perfect DNA markers, Novagen, UK) at 8 V/cm for one hour. DNA was visualised under UV light using a transilluminator and the result recorded photographically.

#### **2.1.10. Nucleotide sequencing**

Clones with inserts were purified using the QIAquick PCR Purification Kit (Qiagen, UK) and the nucleotide sequence of the insert DNA was determined by DBS Genomics at Durham University. The primers 5'-gtaatacgactcactataggcg-3' and 5'-gctatttagtgacactatag-3' were used for sequencing inserts within the pGEM-T vector, while primers 5'-cgactcactatagggagacc-3' and 5'-cgcttttgagaggggagtactc-3' were used for sequencing inserts within the pIRESblast vector. Sequences were compared with the GenEMBL data bank entry using the Bestfit program from the GCG package (University of Wisconsin Genetics Computer Group sequence analysis software).

#### **2.1.11. Purification of DNA from agarose gels**

Clones verified as correct had their inserts removed by digestion with the appropriate restriction enzymes (Section 2.1.8.). The insert was separated from pGEM-T by agarose gel electrophoresis, gel extracted and finally ligated into the mammalian vector, pIRESblast (described below in Section 2.2.3.2.).

A slice of gel containing the DNA fragment was excised using a sterile scalpel and the DNA extracted from the gel using a commercial kit (QIAquick Gel Extraction Kit,

Qiagen, UK) based on silica-gel membrane binding. The DNA was eluted in 30 µl of sterile water.

**2.1.12. Dephosphorylation of plasmid DNA**

When DNA was prepared for ligation, the 5' ends of the parent plasmid were dephosphorylated in order to prevent religation. The 5' ends of the insert were left intact, so favouring their integration. Restriction digested plasmid DNA (10 µl) was dephosphorylated by incubation with 3.75 units calf intestinal alkaline phosphatase (Amersham biosciences, UK) at 37°C for 1 hour and then heat-inactivated at 85°C for 10 minutes.

**2.1.13. Ligation of DNA fragments into a plasmid**

Ligations were carried out in 10 µl reaction volumes.

A typical ligation reaction was as follows:

Reagent	Volume (µl)
Parent plasmid digested with appropriate restriction enzymes and phosphatase-treated (50 ng/µl)	1
Gel-extracted fragment (Section 2.1.11.)	3
One-Phor-All (OPA) buffer (Amersham Biosciences, UK)	1
Sterile H <sub>2</sub> O	3
ATP (10 mM)	1
T4 DNA ligase (Promega, UK) diluted to an initial concentration of 0.5 U/µl	1

The first four reagents were placed in a tube and heated to 65°C for 5 minutes then cooled on ice in order to break the weak hydrogen bonds between compatible ends



and allow new ones to form between the plasmid and the insert. The final two reagents were then added and the tube left at 10°C overnight.

Control reactions were also included in which insert DNA was replaced with sterile water.

The next day, ligation reactions were transformed into JM109 cells (Section 2.1.5.).

#### **2.1.14. Site-directed mutagenesis (SDM)**

Site-directed mutagenesis - a molecular biology technique which creates a mutation at a specified site within a plasmid DNA molecule – was carried out using the following approach:

two oligonucleotide primers (of approximately 30 bp), each complementary to opposite strands of a plasmid containing the insert of interest, were designed which contained the desired mutation, and a typical reaction was set up in a thin-walled 500 µl PCR tube.

Reagent	Volume (µl)
Primer 1 (25 ng/µl)	5
Primer 2(25 ng/µl)	5
Sterile H <sub>2</sub> O	30
PfuTurbo (2.5 U/µl, Stratagene)	1
dNTP mix (1.25 mM)	8
Template (5-50 ng/µl)	1

The PCR tube was then placed in a Techne Genius thermal cycler (Techne Ltd, Cambridge, UK) set to run as follows:

Segment	Cycles	Temperature	Time
1*	1	95°C	30 seconds
2	12-18	95°C 55°C 68°C	30 seconds 1 minute 1 minute/kb of plasmid length

\*Plasmid template was added following this segment.

PfuTurbo is an extremely high fidelity DNA polymerase which permits the synthesis of DNA strands that anneal to form a plasmid. This plasmid is identical to the original except for the single desired mutation and the fact that the DNA is no longer methylated (plasmids are methylated by most strains of bacteria). The latter point was crucial to the next step, which involved incubating the reaction mixture with *DpnI*, a restriction enzyme that digests DNA at the relatively common GATC site but only if it is methylated. The majority of the original unmutated plasmid DNA was therefore cleaved whereas plasmids created by polymerase primer extension and containing the mutation were left intact and in a form capable of being transformed into JM109 bacteria (Section 2.1.5.).

This technique was used to make silent mutations in genes of interest in order to remove restriction enzyme-cleavable sites that were required for subcloning steps. SDM progress was monitored by restriction digestion, the enzyme concerned being used to differentiate between the wild-type plasmid and successfully mutated DNA. See Fig. 2.1.2. for an example of an *EcoRI* site having been removed by SDM and detected following *EcoRI* digestion by visualisation under UV light.

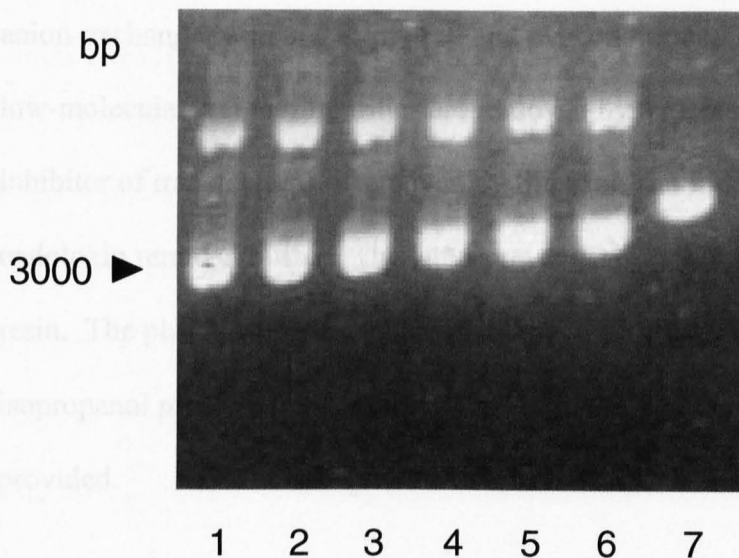


Fig. 2.1.2. Restriction endonuclease analysis of small scale preparation of plasmid DNA aimed at establishing whether mutation by SDM had been successful.

Since, in this case, SDM was used to remove an internal *EcoRI* restriction site, digestion was performed with *EcoRI* restriction enzyme.

The first six lanes contain putative clones 1 to 6, lane 7 the original unmutated plasmid. The conclusion was therefore that all clones had had their *EcoRI* sites successfully removed.

### **2.1.15. Endotoxin-free large-scale preparation of DNA**

When a vector construct had been created and confirmed by sequencing as being correct, a large-scale preparation was performed using the Endofree Plasmid Maxi Kit (Qiagen). This kit uses a modified alkaline lysis method (Birnboim & Doly, 1979; Ish-Horowicz & Burke, 1981), followed by the binding of plasmid DNA to anion-exchange resin under low salt and pH conditions. All other RNA, proteins and low-molecular weight impurities are removed by a medium salt wash. Endotoxin (an inhibitor of transfection) is removed by filtration and incubation on ice with an endotoxin removal buffer. The latter prevents any endotoxin from binding to the resin. The plasmid DNA is eluted in a high salt buffer, concentrated and desalted by isopropanol precipitation, and dissolved in 0.5 ml of the endotoxin-free buffer provided.

### **2.1.16. Determination of DNA concentration and purity**

DNA was quantified by UV absorbance, assuming an O.D. at 260nm of 1.0 was equal to 50 µg/ml. Absorbance measurements were taken using a quartz cuvette (Scientific Supplies Co.) with a 1 cm path length in a Philips PU8720 UV/VIS spectrophotometer.

The A<sub>260</sub>/A<sub>280</sub> nm ratio was taken as an index of DNA purity, i.e. the extent to which DNA was contaminated with protein. Only DNA with a ratio >1.8 was used in transfections.

## **2.2. Cell culture techniques**

### **2.2.1. The Epo-dependent erythroleukaemic AS-E2 cell line**

The Epo-dependent erythroleukaemic AS-E2 cell line (Miyazaki *et al*, 1997) was kindly donated by Drs Tomonaga and Imai (Nagasaki University, Nagasaki, Japan).

### **2.2.2. Maintenance of cell lines**

#### **2.2.2.1. Tissue culture reagents**

Cells were cultured under aseptic operating conditions and in sterile growth media.

The media used in these studies provide the cells with optimum growth conditions for extracellular survival.

Iscove's Modified Dulbecco's Medium (IMDM, Sigma, UK) was supplemented with Epo, foetal calf serum (FCS), glutamine and antibiotics. The FCS provides growth factors, while antibiotics minimise the risk of bacterial contamination.

*Medium used to culture AS-E2 cells, 200 ml:*

Reagent	Volume (ml)	Final concentration
IMDM	154 ml	-
Epo (20,000 IU/ml)	20 µl	2 IU/ml
FCS	40 ml	20%
Penicillin (10,000 U/ml)	2 ml	100 U/ml
Streptomycin (10 mg/ml)	2 ml	100 µg/ml
Glutamine(200 mM)	2 ml	2 mM

The cells were passaged every 3-4 days and seeded at an initial density of

$5 \times 10^4$  cells/ml.

### **2.2.2.2. Maintenance and culture of cell lines**

Cell cultures were maintained in tissue culture incubators at 37°C in an atmosphere of 5% CO<sub>2</sub>/95% air. The growth medium was replaced every 3-4 days. In the case of the AS-E2 cell line, cells were centrifuged, the supernatant poured off and the pellet resuspended in approximately 2 ml of fresh medium. A sample (20 µl) was taken for cell counting, using a Neubauer haemocytometer.

### **2.2.2.3. Storage and recovery of cells**

Cells were suspended in cryopreservant, full medium supplemented with dimethylsulphoxide (DMSO, Sigma, UK), at a concentration of 10% (v/v) and pipetted into 2 ml cryotubes. The tubes were placed in a polystyrene cell freezing box and left overnight in a -70°C freezer. The polystyrene acts as an insulator, slowing down the freezing process and preventing any extreme shock to the cells. Lastly, the cryotubes were transferred into liquid nitrogen (-196°C) for long-term storage. Cells were retrieved for culture by placing a cryotube into a water bath at 37°C to rapidly thaw the cells. In order to remove DMSO, the contents of the cryotube were added to 10 ml of full medium and centrifuged at 300 g; the supernatant was then removed and the cell pellet resuspended in 12.5 ml of fresh full medium. This cell suspension was then added to a 25 ml flask and placed in the incubator.

### **2.2.3. Transfections**

The ability of a range of transfection reagents to transfect the Epo-dependent cell line AS-E2 was screened using a luciferase-based reporter plasmid (pGL3, Promega) and assayed for luciferase activity (by Promega's Luciferase Assay System) in a Turner luminometer. GeneJuice transfection reagent (Novagen) consistently produced the

highest readings and so was selected for use during this study. GeneJuice consists of a non-toxic cellular protein and a small quantity of a novel polyamine.

#### **2.2.3.1. Method for transfecting AS-E2 cells**

AS-E2 cells were seeded at  $1 \times 10^5$  cells/ml the day before transfection. On the day of transfection they were seeded at  $0.2 \times 10^6$  cells/ml as 3 ml per well of 6-well plates (Falcon, BD Biosciences Catalog #353046). The cells of each well were then transfected with 200  $\mu$ l IMDM medium, 6  $\mu$ l Genejuice and 2  $\mu$ l plasmid DNA at a concentration of 1  $\mu$ g/ $\mu$ l.

#### **2.2.3.2. Vector used for obtaining stable transfections - pIRESblast**

To obtain stably transfected cells that overexpress the protein of interest, cells were transfected with the pIRESblast vector containing the relevant gene.

The pIRESblast vector was created by excising the neomycin gene from pIRESneo as a XmaI/XhoI fragment and replacing it with a blasticidin resistance cassette, which was derived from pcDNA 6.2-DEST Gateway (Invitrogen).

*The blasticidin-resistance cassette was PCR-amplified with primers incorporating the appropriate restriction ends:*

Name	Function	Sequence	Restriction Site
BlastXmaI-5'	Forward primer for amplifying the blasticidin resistance gene and the SV40 early polyadenylation signal	5'-gacgat <u>cccg</u> ggc gacaaggtgaggaact aacc-3'	- <i>Xma</i> I underlined
BlastXhoI-3'	Reverse primer for amplifying the blasticidin resistance gene and the SV40 early polyadenylation signal	5'-gacgat <u>ctcg</u> agc agacatgataagatac attgatgagt-3'	- <i>Xho</i> I underlined

The pIRESblast vector contains an Internal Ribosome Entry Site (IRES) sequence which permits the translation of two open reading frames from one mRNA (see Fig. 2.2.1. for a map of the vector).

### 2.2.3.3. Blasticidin selection

Two days after transfection, and allowing time for expression of the blasticidin-resistance gene, the cells were reseeded into flasks at a concentration of  $5 \times 10^4$  cells/ml with blasticidin (10  $\mu$ g/ml). For approximately two weeks the cells were passaged as normal, every 3-4 days, with reseeded at  $5 \times 10^4$  cells/ml but continuing with blasticidin pressure. During this period, however, blasticidin-induced cell-death meant a reduction in cell numbers, the latter falling to 500,000 cells (in 10 ml of medium). Cells then began to grow back and soon reverted to their original doubling time of approximately two days. Similarly treated, but untransfected, cells did not survive beyond day 7.



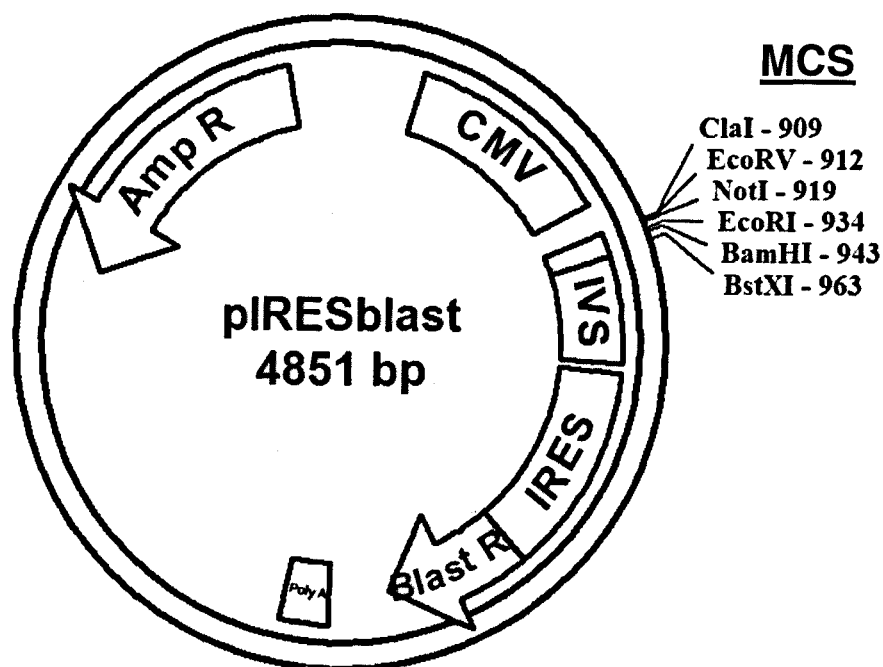


Fig. 2.2.1. Map of pIRESblast vector used in mammalian transfections. The DNA sequence is inserted into the multiple cloning site (MCS). The vector contains the blasticidin-selectable marker (Blast R), a cytomegalovirus (CMV) enhancer-promoter for high-level expression and an ampicillin resistance gene that allows selection and maintenance in *E. coli*.

In some cases cells did not grow back and so, when the cell number reached ~50,000, they were added (as a 1 ml suspension) to 8 ml of Clonacell (StemCell Technologies, UK) and 1 ml of IMDM containing 10x glutamine, 10x antibiotics, 10x Epo and 10x blasticidin to give final 1x concentrations. Clonacell is a methylcellulose-based semi-solid medium which allows suspension cells to grow as discrete colonies that can then be individually picked by pipette. The above mixture was syringed into 10 ml cell culture plates and placed in a box with a loose-fitting lid containing a lidless cell culture plate filled with sterile water to maintain humidity. The box was incubated for approximately two weeks, by which point individual cell colonies could be seen, picked in 20 µl volumes and grown up in 96-well plates (200 µl blasticidin-containing full medium per well).

#### **2.2.3.4. PCR-amplification from cell extract**

PCR-amplification was used to determine whether transfected blasticidin-resistant cell lines contained recombinant DNA.

Mammalian cells were centrifuged, washed once with PBS (see Appendix), re-centrifuged and suspended in K buffer (0.5% Tween 20 [v/v], 100 µg/ml proteinase K, from Sigma, in 1x Expand High Fidelity buffer) at a final concentration of 5000 cells/µl. The suspension was then heated at 56°C for 45 minutes. The resulting cell extract (2 µl) was used in a 50 µl volume PCR reaction.

PCR-amplification of human  $\beta$ -actin (GenEMBL Accession No. X00351) with actin-5' (TGACGGGGTCACCCACACTGTGCCCATCTA) and actin-3' (CTAGAAGCATTGTGCGGTGGACGATGGAGGG) primers was used as a positive control.

#### **2.2.4. AS-E2 Epo bioassays**

Epo-induced proliferation in AS-E2 cells was measured using alamarBlue (Serotec Ltd, UK). Before commitment to bioassay the AS-E2 cells were washed three times in IMDM and maintained as stock cultures for 72 hrs in Epo-free IMDM containing 5% FCS. The AS-E2 cells were suspended in IMDM at a density of  $2.5 \times 10^6$  cells/ml. The cell suspension (100  $\mu$ l/well) was transferred to a 96-well microtitre plate (Falcon, Catalogue No. 353072). Epo intended for testing was diluted in assay medium consisting of IMDM, 200 units/ml penicillin and 200  $\mu$ g/ml streptomycin and then added to the wells as an additional 100  $\mu$ l. Epo-free assay medium was used in negative control wells. Concentrations were expressed as final concentrations in the wells (nM). The assays were done in triplicate, using serial twofold dilutions. Only the inner 60 wells were used, owing to the outer wells' greater susceptibility to so-called "edge effects", widely thought to be caused by the outer wells being exposed to more rapid variations in temperature and pH (Meager, 2002). The cells were incubated for one week under the conditions described above. Their reducing activity was assayed by adding 20  $\mu$ l of alamarBlue solution, followed by a further 24-hour incubation period. The bioassay responses of the 96 wells were quantified with a SpectraMax 340PC or an E-max precision microplate reader (both from Molecular Devices Corporation, Sunnyvale, California, US) measuring optical densities at 570 and 600 nm. The values were calculated using SOFTmax (Molecular Devices Corporation) software.

#### **2.2.4.1. Statistical analysis**

The results were expressed as mean and standard error of the mean (s.e.m). Median effective concentration ( $EC_{50}$ ) values (in other words the concentration required to induce a 50% effect) were calculated by non-linear regression and, using GraphPad Prism for Windows, version 4.00 (GraphPad Software, San Diego, California, USA), were assigned 95% confidence intervals (probability (P) values less than 0.05 were regarded as statistically significant). Comparisons between sample dose-response curves were based on comparison of the estimated  $EC_{50}$  values. For added rigour, tight clusters of results were also analysed, using either the in-house WRANL program featuring iterative weighted regression analysis of logistically transformed responses on log dose (Gaines & Tydeman, 1982) or RANDOM which analyses only the steepest part of the graph. COMPOT was a program used to calculate combined estimate of potencies. Pearson correlations between results were performed using Minitab. R values greater than 0.7 were considered as positively correlated and R values lower than -0.7 were considered as negatively correlated, these being commonly used thresholds for strong statistical similarity.

### **2.3. Protein Analysis**

#### **2.3.1. Preparation of cell lysates for Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis**

Cells from 50 ml of culture were centrifuged and resuspended in lysis buffer (~100  $\mu$ l – see Appendix for buffer recipe).

### **2.3.2. Measurement of protein content and preparation of samples for electrophoresis**

Total protein was determined using the bicinchoninic acid (BCA) assay (Pierce, Cheshire, UK), which is based on measuring  $\text{Cu}^+$  ion concentration (Smith *et al*, 1985). Samples were mixed with 6x SDS loading buffer (see Appendix) in the ratio 5:1, incubated for 5 min at 95°C and resolved by SDS-PAGE (Section 2.3.3.).

### **2.3.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (adapted from Laemmli, 1970)**

*Recipe for the preparation of SDS-PAGE gels:*

Reagents	Separating gel 11%	Stacking gel 5%
30% (w/v) Acrylamide: 0.8% (w/v) Bis-Acrylamide (National Diagnostics, UK)	4.5 ml	1.0 ml
Tris-HCl pH 8.8 (1.85 M)	2.8 ml	-
Tris-HCl pH6.8 (1.25 M)	-	0.6 ml
MilliQ water	5.0 ml	4.3 ml
10% SDS	140 $\mu\text{l}$	60 $\mu\text{l}$
N,N,N',N'- Tetramethylethylenediamine (TEMED)	20 $\mu\text{l}$	10 $\mu\text{l}$
10% ammonium persulphate	85 $\mu\text{l}$	30 $\mu\text{l}$

The electrophoresis apparatus was set up in accordance with the manufacturer's specifications (Mini-Protean II, Bio-Rad, UK). SDS running buffer (800 ml) was added to the tank (see Appendix for recipe).

Protein samples (20  $\mu$ l) were loaded against 10  $\mu$ l of protein ladder (BenchMark Pre-Stained Protein Ladder, Invitrogen, UK) and the samples run at 25 mA per gel for 1.5 hours.

#### **2.3.4. Protein transfer**

Before transfer, a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, UK) was soaked in 100% methanol for 1 minute, rinsed with water and immersed in blot transfer buffer (see Appendix for recipe).

The transfer sandwich was set up in the following order: the anode plate, four pieces of blotting paper cut to the size of the SDS-PAGE gel, the PVDF membrane, the SDS-PAGE gel, four more pieces of blotting paper, and finally the cathode plate. The current was set at 200 mA for 2 hours adapted from (adapted from Towbin *et al*, 1979).

#### **2.3.5. Western blotting**

Following transfer, membranes were blocked with 5% (w/v) milk powder/0.1% (v/v) Tween-20 in phosphate-buffered saline (PBS-milk) for 1 hour at room temperature with gentle agitation (recipe for PBS same as that used in Section 2.2.3.4).

Membranes were incubated overnight at 4°C with an antibody (1/1000 dilution in PBS-milk) directed against the protein of interest. After antibody binding, membranes were further washed in PBS containing 0.1% Tween-20 (PBS-Tween) for 3 x 30 minutes with gentle agitation. Membranes were then incubated for 1 hour at room temperature with a secondary antibody (1/1000 dilution PBS-milk) that binds specifically to the bound primary antibody. Membranes were washed for 3 x 30 minutes in PBS-Tween with gentle agitation. The secondary antibody is

conjugated to alkaline phosphatase. Specific protein bands can then be detected using the colorimetric alkaline phosphatase substrate 5-bromo-4-chloro-3 indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Sigma, UK).

**2.3.6. Isoelectric focusing**

Isoelectric focusing (IEF) was performed by polyacrylamide slab gel electrophoresis on a Bio-Rad Protean 2 16 cm slab cell.

*Recipe for the preparation of an IEF gels:*

Reagent	Volume (to make one gel)		Final concentration
72.5% (w/v) Acrylamide: 2.5% (w/v) Bis-Acrylamide (Bio-Rad, UK)	7 ml		10.0% (w/v) Acrylamide; 0.34% (w/v) Bis-Acrylamide
Sucrose (60% [w/v], Sigma, UK)	12.5 ml		14.8% (w/v)
MilliQ water	28 ml		-
	For pH 2.5-9.0	For pH 2.5-7.0	2.5%
Ampholines pH 3-10 (all ampholines from Sigma, UK)	0.6 ml	-	
Ampholines pH 2.5-5	0.22 ml	0.625	
Ampholines pH 5-7	0.22 ml	0.625	
Ampholines pH 7-9	0.22 ml	-	
Riboflavin (0.0004% [w/v], Sigma, UK)	2 ml		0.000016%
N,N,N',N'- Tetramethylethylenediamine (TEMED, Bio-Rad, UK)	14 µl		0.00028%

The anode buffer and cathode buffer consisted of 10 mM orthophosphoric acid and 20 mM sodium hydroxide respectively.

Sucrose was added to the Epo samples to reach a final concentration of 20% and 10 µg loaded per well. Isoelectric focusing markers from Serva (pH 3-10) and from Amersham Biosciences (pH 2.5-6.5) were used to enable determination of the isoelectric point (pI) of bands. Gels were run overnight at a constant 340 V and, the following morning, either silver stained or transferred to membrane.

### **2.3.7. Silver staining**

Incubations were carried out on an orbital shaker. Solutions were drained off between each step. The proteins in the gel from Section 2.3.6. were first precipitated by incubation in a 10% (w/v in MilliQ water) solution of trichloroacetic acid. Solution A (200 ml, see appendix for recipe) was added to the gel for five minutes, drained off and the remaining 250 ml was then added to the gel for two hours to fix the gel. Four changes of MilliQ water (2 x 200 ml at 60°C and 2 x 200 ml at room temperature) were then added for 15 minutes at a time to wash out the fixative. A 32 µM solution of the reducing agent dithiothreitol (developer) was added for 30 minutes. Silver nitrate solution (0.1% w/v in MilliQ water) was then added for 30 minutes. Solution B (120 ml, see Appendix for recipe) was added until the liquid started to turn yellow, drained, and another 120 ml of Solution B added for 30 seconds, drained, and the remaining 260 ml of Solution B added and shaken until the staining of the bands developed. The staining process was terminated by the addition of citric acid solution (6.05 g in 12.5 ml MilliQ water). After 15 minutes, the gel was then washed several times with MilliQ water.



### **2.3.8. Protein transfer (specific to IEF gels)**

Gels were transferred onto a PVDF membrane (Immobilon-P, Millipore, UK) in 0.7% acetic acid blotting solution for 3 hours at a constant current of 200 mA with the gel/membrane assembled in reverse order so that the membrane was on the cathode side of the gel. This protocol is recommended for the transfer of proteins with a high pH. The membrane was then processed as in Section 2.3.5. Once colour had been developed using BCIP/NBT, the blot was read by densitometer as described in Section 2.3.9. below.

### **2.3.9. Densitometric analysis**

Gels and blots were scanned by densitometer (Molecular Dynamics Personal Densitometer SI). Densitometric analysis of the bands was performed using ImageQuant software (Molecular Dynamics ImageQuant, Sunnyvale, California, USA). The amount of each Epo isoform was expressed as a percentage of the total staining intensity of all bands. The mean pI value of a sample was calculated using the following equation:

$$\text{Mean pI value} = \sum (pI_i) \left( \frac{x_i}{\sum x_i} \right)$$

where  $pI_i$  is the pI of the  $i^{\text{th}}$  band and  $x_i$  is the amount of the  $i^{\text{th}}$  band ( $\sum x_i = 100\%$ ).

### **2.3.10. Desialylation of Epo**

A range of desialylated Epo samples was produced that could be tested during the course of this project. Erythropoietin was dissolved in buffer A (150 mM NaCl, 50 mM sodium acetate, 1 mM  $\text{CaCl}_2$ , pH to 5.5 with HCl) to give a final concentration of 300  $\mu\text{g/ml}$ . Neuraminidase conjugated to agarose gel (314  $\mu\text{l}$  of a 1:1 mixture of gel/buffer A, Calbiochem) was added to 6 ml of the above Epo solution, then mixed

by slow vertical rotation (12 rpm) at 37°C. Equal volume aliquots (570 µl) were removed at t=0, 5, 10, 20, 40, 80, 160, 480, 960 and 1800 min, centrifuged to remove the neuraminidase-agarose and stored at -40°C.

### **2.3.11. Ion-exchange high performance liquid chromatography (HPLC)**

Preparative ion-exchange HPLC was performed using a Gilson HPLC system. Epo (Amgen, lot 5141) was diluted tenfold from an original concentration of 3.19 mg/ml in 0.01 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0. This solution (5 ml) was loaded onto a column (MonoQ, Pharmacia) at 3 ml/min in Buffer A (0.01 M sodium phosphate pH7.0, 0.025 M NaCl). After three minutes, this process was repeated a second time, whereupon the run was started at a flow rate of 2 ml/min equilibrated in Buffer A, following the absorbance of the eluate at 276 nm.

*The following ramp was used:*

Time / min	%A (0.01M sodium phosphate pH7.0, 0.025M NaCl)	%B (0.01M sodium phosphate pH7.0, 1M NaCl)
0	100	0
5	100	0
45	80	20
46	100	0
60	100	0

Ten x 2-minute fractions (4 ml/fraction) were collected, followed by 0.4 min fractions (0.8 ml/fraction).

### **2.3.12. Enzyme-linked immunosorbent assay (ELISA)**

Epo concentrations were determined using an anti-Epo ELISA (generously donated by B. Rafferty, Division of Immunobiology and Endocrinology, NIBSC).

Nunc MaxiSorp ELISA plates (Nunc, Roskilde, Denmark) were coated with rabbit anti-Epo antibody (200  $\mu$ l per well, 5  $\mu$ g/ml in PBS) and incubated overnight at 4°C. The following day plates were washed three times with wash dilution buffer (WDB, see Appendix for recipe).

Serial dilutions of samples for testing and a standard ( $2 \times 10^{-10}$  g/ml as the top dose) made up in WDB were added as 100  $\mu$ l. Biotinylated goat anti-Epo antibody (100  $\mu$ l, 7  $\mu$ g/ml, B. Rafferty), diluted in WDB with 1% goat serum, was also added to the wells. The plates were incubated for four hours with shaking at room temperature, then washed three times with WDB. Horse Radish Peroxidase-conjugated Avidin in WDB (200  $\mu$ l, 0.025% [v/v], DAKO) was then added for 15 minutes and washed three times with WDB. *O*-Phenylenediamine dihydrochloride (OPD, Sigma), a horse-radish peroxidase substrate (0.02% [w/v]) and hydrogen peroxide ( $H_2O_2$ , 0.04% [v/v]) were added to substrate buffer (see Appendix) and 200  $\mu$ l added to the wells.

Sulphuric acid ( $H_2SO_4$ , 100  $\mu$ l, 1 M) was added to stop the chromogenic reaction.

The absorbance was read at 490 nm on the SpectraMax 340PC (Molecular Devices Corporation, Sunnyvale, California, US).

Relative concentrations were determined using the in-house program WRANL (Iterative weighted regression analysis of logistically transformed responses on log dose (Rybicki & von Wechmar, 1982).

### **2.3.13. Fluorescence microscopy**

Coverslips were coated with poly-L-lysine (Sigma, UK) at room temperature for 15 minutes then left to dry. Cells ( $2 \times 10^6$ ) in a volume of 200  $\mu$ l were placed on the coverslip and allowed to adhere for one hour at 37°C. Excess cells were blotted off and the remaining cells fixed by the addition of 4% (w/v) formaldehyde in PBS for 30

minutes at room temperature. The cells were washed three times with PBS.

Alpha-1-acid glycoprotein, desialylated for 48 hours as in Section 2.3.10., and conjugated to Alexa Fluor 488 (Molecular Probes, UK) according to the manufacturer's instructions, was added to the cells in a 200  $\mu$ l volume (8  $\mu$ g/ml in PBS) and incubated for 20 minutes at room temperature. Coverslips were washed three times with PBS.

To stain the actin cytoskeleton, a solution of a fluorescence-conjugated actin-binding toxin, Phalloidin-594 (Molecular Probes, UK), was made up in PBS (10  $\mu$ l plus 170  $\mu$ l) from a 200 unit/ml (dissolved in methanol) stock, added to the coverslip and incubated for 40 minutes at 37°C. Salts were removed by three washes with MilliQ water, whereupon the nuclei of the cells were stained for 30-40 seconds with Hoechst 33342 (Molecular Probes, UK), a dye which binds adenine-thymidine base pairs. After coverslips were rinsed twice with MilliQ water, cells were permanently mounted in Vectashield mounting medium (Vector laboratories, UK).

Confocal laser scanning microscopy (CLSM) was performed by Drs L. Berry and R. Fleck of NIBSC with a Leica SP2 AOBS confocal laser scan head mounted on an upright microscope (DM RE-7 Leica) set up for dual channel fluorescence using fluorescein and Alexa594 filter settings (laser wavelength 488 nm and 543 nm). Images were taken using a 20x lens (NA 0.70) or an oil-immersion objective (HCX PL Apo  $\lambda$ BL NA 1.4-0.6) and separated with spectral filtering (user optimised) for each fluorophore and collected by sequential scanning exciting each fluorophore individually. Images were analysed and exported using the Leica 3D software package.

#### **2.3.14. Glycoanalysis (carried out by Dr Yuen, NIBSC)**

N-Glycans were first released from Epo by peptide:N-glycosidase F and labelled with the fluorescent label, 4-aminobenzoic acid (Yuen *et al*, 2002). The labelled N-Glycans were then resolved by normal-phase HPLC and plotted on a graph. Identification of the fractionated derivatised oligosaccharides within each peak was determined by Matrix-assisted laser desorption ionization (MALDI) mass spectrometry using a MALDI mass spectrometer (Micromass TofSpec-2E, Macclesfield, UK). The percentages of each oligosaccharide identified within a fraction were calculated by measuring the area under the relevant peak.

### **2.4. In vivo bioassays: the post-hypoxic polycythaemic and normocythaemic mouse assays for Epo**

In line with Institute policy, all animal handling was carried out by professional animal technicians in Biological Support Services, NIBSC, in particular by Peter Gerson and Pauline Lloyd. All *in vivo* experiments were performed according to UK Home Office regulations and were approved by the Local Ethics Committee.

The post-hypoxic polycythaemic and the normocythaemic mouse assays for Epo are the only two assays currently recognised by the European Pharmacopoeia as being capable of measuring the *in vivo* biological activity of Epo.

#### **2.4.1. The post-hypoxic polycythaemic mouse assay for Epo**

During the course of this assay, mice are rendered polycythaemic by exposure to reduced atmospheric pressure. Endogenous production of Epo is reduced, making them sensitive to the hormone. The biological activity of Epo is then estimated by measuring the incorporation of  $^{59}\text{Fe}$  into circulating red blood cells.

Female CBA/Ca mice (16-23 g, purchased from Harlan UK Limited) were placed in a hypoxic chamber at 0.6 atmospheres on Day 0. On Day 3, the atmospheric pressure was reduced to 0.4 atmospheres. On Day 14, the mice were returned to normal atmospheric conditions. On Day 14+3, sequential dilutions of the Epo samples intended for testing and the standard were made up in Albumin-buffered saline solution pH7.2 (ABS – see Appendix for buffer composition) at concentrations of approximately 600, 300 and 150 mIU/ml. Doses (0.2 ml) were injected subcutaneously into the mice. Blanks consisted of ABS alone. Six mice were used per treatment group. On Day 14+5, 0.2 ml of  $^{59}\text{Fe}$  (1  $\mu\text{Ci/ml}$ ) in citrate buffer (see Appendix) was injected intraperitoneally. On Day 14+7, mice were injected intraperitoneally with 0.3 ml of the anaesthetic Avertin (10 g of 2,2,2 tribromoethanol in 10 mls 2 methyl 2 butanol, BDH). A blood sample was taken from the orbital sinus of each mouse into a capillary tube which was then sealed with Cristaseal (Hawksley & Sons Ltd, UK) and centrifuged for five minutes, enabling measurement of the percentage of red blood cells in the total blood volume (the haematocrit or packed cell volume). Blood samples (0.5 ml) were taken by heart puncture, then measured by gamma counter (LKB1282).

The response was calculated for each mouse as percentage of dose of  $^{59}\text{Fe}$  incorporated into the total blood volume of the mouse (assumed to be 7.5% of body weight).

$$\text{Response} = \frac{{}^{59}\text{Fe in blood sample (cpm)} \times \text{body weight (g)} \times 7.5}{\text{Volume of blood sample (ml)} \times {}^{59}\text{Fe dose (cpm)}}$$

#### **2.4.2. The normocythaemic mouse assay for Epo**

Female Balb/c mice (17-21 g) were purchased from Harlan UK Limited. On Day 1, sequential dilutions of the Epo samples intended for testing and the standard were prepared in Albumin-buffered saline solution pH7.2 (ABS) at concentrations of approximately 50, 100 and 200 IU/ml. Doses (0.2 ml) were injected subcutaneously into each mouse. Blanks consisted of ABS alone. Six mice were used per treatment group. On Day 5, all the mice were anaesthetised by intraperitoneal injection with 0.3 ml Avertin (BDH). Blood (approximately 20  $\mu\text{l}$ ) was taken up from the orbital sinus of each mouse and placed in a heparinised tube. Reticulocyte concentration - determined by adding 5  $\mu\text{l}$  of the blood samples to 1 ml of RetiCount (a fluorescent dye that binds the RNA of immature red blood cells, Becton Dickinson) and incubating for at least 30 minutes in the dark - was then analysed by FACScan (FACScalibur, Becton Dickinson).

##### **2.4.2.1. Statistical analysis of in vivo results**

All bioassay estimates were expressed in terms of the Second International Standard for Erythropoietin, NIBSC Code: 88/574 (Storring & Gaines Das, 1992).

After the log dose-log response data were analysed as paired parallel-line assays, an analysis of variance was performed for each assay. Statistical weights were calculated

as the reciprocal of the variance of the log potencies. Estimates of log potency were examined for heterogeneity using a  $\chi^2$  test. Homogeneous estimates ( $P > 0.05$ ) were combined as weighted geometric means, and heterogeneous estimates were combined as unweighted geometric means with fiducial limits based on the variance of the logarithms of the combined estimates.



## **Chapter 3. A sialylation-sensitive cell-based *in vitro* bioassay**

### **for erythropoietin; incorporation of the galactose-binding**

#### ***Erythrina crista-galli* lectin.**

#### **Summary and overview**

This chapter describes the development of a sialylation-sensitive *in vitro* assay by incorporation of the galactose-binding properties of a plant-derived lectin into a cell-based Epo bioassay. It features the following sequence of studies:

(i) Development of an *in vitro* bioassay protocol incorporating a solid-phase lectin binding step which differentiates between sialylated and desialylated Epo (Section 3.2.1.);

(ii) Validation studies on three separate sample sets, which are expected to exhibit a range of bioactivities:

-sample set 1: neuraminidase desialylated Epo preparations (Section 3.3.1.)

-sample set 2: Epo preparations generated through different production and purification methods and varying in biological activity (Section 3.3.2.).

-sample set 3: pharmaceutical Epo separated into anion-exchange subfractions, relatively enriched with differentially charged isoforms (Section 3.3.3.);

(iv) Comparisons of *in vitro* results with *in vivo* bioactivity measurements (Sections 3.3.1.8., 3.3.2.4. and 3.3.3.6. (includes correlation studies));

(v) Correlation studies comparing *in vitro* and *in vivo* results with physico-chemical analysis by glycan mapping and IEF (Section 3.3.3.6.);

(vi) Analysis and interpretation of results (Section 3.4.).

### **3.1. Introduction and aims of the study**

Lectins are non-enzymatic proteins that bind to carbohydrates non-covalently. Widely distributed in plants, animals, micro-organisms and bacteria, they are in many cases unrelated structurally, although some common structures exist that have arisen by convergent evolution (Loris, 2002; Sharon, 1993). Animal lectins include: C-type lectins (involved in mediating adhesion, glycoprotein clearance and pathogen neutralization), one of which is the ASGPR; galectins (crosslink sugars in the extracellular matrix); I-type lectins (immunoglobulin-related sialic acid-binding proteins) and P-type lectins (which bind to mannose 6-phosphate and are involved in protein sorting) (Dodd & Drickamer, 2001).

Plants also produce lectins with a wide range of carbohydrate-binding specificities, possibly due to sequence hypervariability in the region responsible for sugar binding (Sharon & Lis, 1990). Although the function of plant lectins is not fully known, investigators are keen to exploit their wide range of abilities in terms of binding to different sugars. Table 3.1.1. lists some commonly used lectins and their binding specificities.

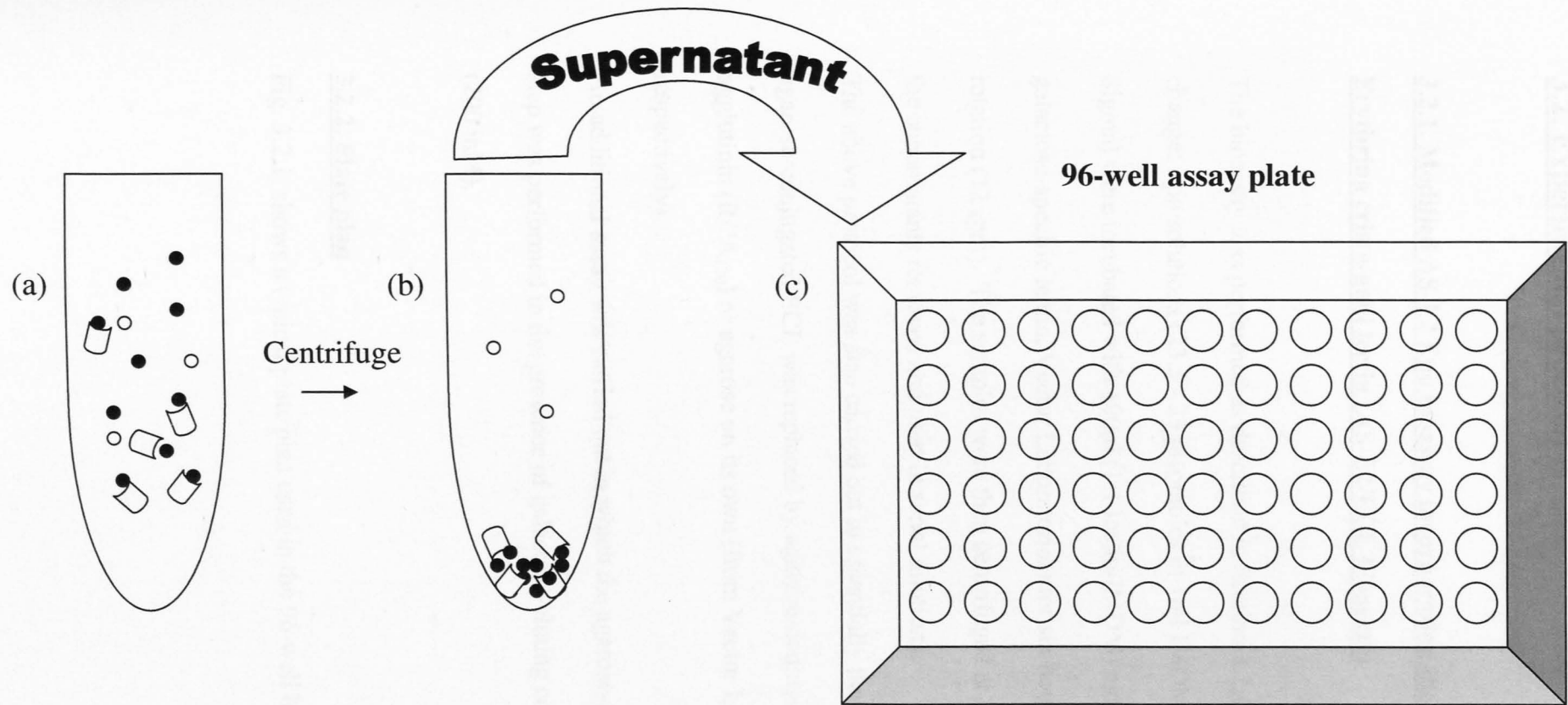
As explained in the introduction, correct glycosylation, including terminal sialylation, is essential for the *in vivo* activity of Epo but has little bearing on its *in vitro* activity (Tsuda *et al*, 1990). The work described in this chapter was aimed at developing a sialylation-sensitive cell-based assay for Epo and investigating how effective it was at assessing potency compared to other *in vitro* assays, physico-chemical tests and the gold standard of *in vivo* assays. One step of the cell-based assay developed exploited the similarity in binding characteristics between ASGP-R and certain galactose-binding plant lectins. The liver's ASGP-R binds glycoproteins containing exposed galactose residues thus removing them from the circulation and preventing

Lectin abbreviation	Plant species from which the lectin is derived	Sugar Ligand
ConA	<i>Canavalis ensiformis</i>	Mannose/Glucose
RCA	<i>Ricinus communis</i>	Galactose
ECL	<i>Erythrina crista-galli</i>	Galactose
DBA	<i>Dolichus biflorus</i>	N-acetylgalactosamine
SNA-1	<i>Sambuccus nigra</i>	Sialic acid

Table 3.1.1. Commonly used lectins, the plant species from which they are derived and their binding specificities (Storring *et al* (1996)).

them from exerting an effect on their target cells. The action of the liver receptor was, in this context, mimicked most successfully by means of *Erythrina crista-galli* lectin (ECL). See Fig. 3.1.1. for an outline of the assay protocol. In essence, a solution of Epo of known concentration was incubated with agarose-conjugated ECL, allowing the lectin to bind Epo containing terminal galactose residues. The sample was then centrifuged, causing complexes comprised of agarose-conjugated ECL bound to such Epo molecules to be pulled down. The supernatant was then used in an Epo-dependent AS-E2 cell bioassay. Epo solutions containing a higher proportion of terminal galactose residues would theoretically be depleted of more Epo and would therefore stimulate a reduced proliferation response in the Epo-dependent assay. Three different types of Epo were tested using the assay developed, each representing one of the different forms of Epo that might require assessment in terms of the quality of Epo as a medicinal product: experimentally desialylated Epo, a range of Epo samples generated by different production and purification procedures, and a batch of pharmaceutical grade Epo separated into fractions by anion-exchange HPLC. These three Epo sample sets were also tested using the unmodified Epo-dependent cell-based and *in vivo* bioassays. Physico-chemical analysis was carried out to allow further investigation of the relationship between glycosylation, in particular, sialylation and galactose exposure, and *in vivo* activity.

Fig. 3.1.1. (Facing page) Schematic representation of the rationale behind the AS-E2/ECL bioassay protocol; (a) a solution of Epo of known concentration is incubated with agarose-conjugated ECL (Vector Laboratories) for 6 hours at 4°C, allowing the lectin to bind any Epo terminal galactose residues, (b) the sample is then centrifuged, causing such complexes to be pulled down, and (c) the supernatant is used in an Epo-dependent AS-E2 cell bioassay.



Agarose-conjugated *Erythrina crista-galli* lectin



Epo containing terminal galactose residues



Epo containing no terminal galactose residues (could be masked by sialic acids)

## **3.2. Experimental procedures**

### **3.2.1. Modified AS-E2 Epo bioassay incorporating the use of agarose-conjugated *Erythrina crista-galli* lectin (AS-E2/ECL bioassay)**

The bioassay was performed as described in Section 2.2.4., but with the following change: Epo solutions (23.5 - 33 picomoles in 1ml IMDM, 1% bovine serum albumin, Sigma) were incubated with 100 $\mu$ l (1:1 lectin/IMDM) agarose-conjugated ECL (a galactose-specific lectin, Vector Laboratories) for six hours at 4°C with slow vertical rotation (12 rpm). The samples were then centrifuged at 200 g for two minutes and the supernatants removed and used in serial dilutions.

The above protocol was also carried out in essentially the same way except that agarose-conjugated ECL was replaced by agarose-conjugated *Ricinus communis* agglutinin (RCA<sub>120</sub>) or agarose on its own (from Vector Laboratories and Sigma respectively).

An additional assay was carried out in which the agarose-conjugated ECL incubation step was performed in the presence of galactose-eluting concentrations of lactose (200 mM).

#### **3.2.2. Plate plan**

Fig. 3.2.1. shows a typical plate plan used in the 96-well bioassays.

	1	0.5	0.25	0.125	0.0625	0.0625	0.125	0.25	0.5	1	
	1	0.5	0.25	0.125	0.0625	0.0625	0.125	0.25	0.5	1	
	1	0.5	0.25	0.125	0	0	0.125	0.25	0.5	1	
	1	0.5	0.25	0.125	0	0	0.125	0.25	0.5	1	
	1	0.5	0.25	0.125	0.0625	0.0625	0.125	0.25	0.5	1	
	1	0.5	0.25	0.125	0.0625	0.0625	0.125	0.25	0.5	1	

Fig. 3.2.1. Plate plan for cell-based Epo bioassays. Each colour represents a different sample being tested. The top dose (1) corresponds to 0.66 nM. Grey outer wells contain medium only.



### **3.2.3. *In vivo* mouse assay for Epo**

The *in vivo* post-hypoxic polycythaemic and normocythaemic mouse assays for Epo were carried out (Sections 2.4.1. and 2.4.2. respectively) for the purposes of comparison with the *in vitro* assays developed in the course of this project.

### **3.2.4. Glycoanalysis**

Glycoanalysis was carried out on the anion-fractionated Epo samples of Section 3.3.3. by Dr Yuen of NIBSC, as described in Section 2.3.14.

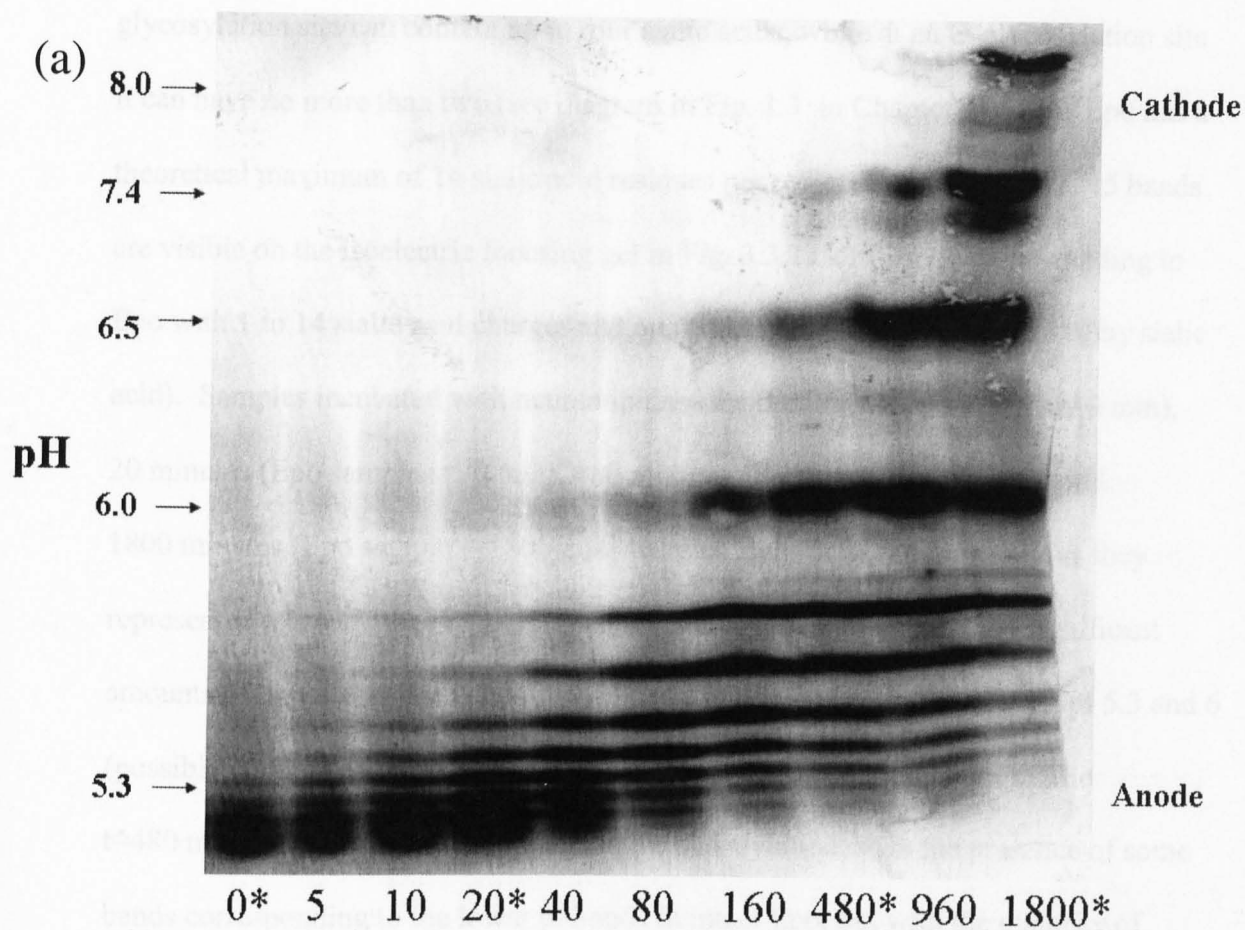
## **3.3. Results**

### **3.3.1. Sample set 1: neuraminidase-desialylated Epo**

#### **3.3.1.1. Desialylation of Epo**

A range of increasingly desialylated Epo samples was generated for use in comparing *in vivo* and *in vitro* assay results. This was achieved by neuraminidase digestion as described in Section 2.3.10. Removal of negatively charged sialic acids increases the pI of a protein, allowing digestion to be followed by IEF (in this case, migration shifts towards the cathode). The samples were run on an IEF gel (ampholines pH3-10:2.5-5:5-7:7-9=1:1:1:1) that was then transferred to a PVDF membrane and Western-blotted with an anti-Epo antibody (generously donated by B. Rafferty, NIBSC) as described in Sections 2.3.5., 2.3.6 and 2.3.8. Fig. 3.3.1a shows an increase in the pI of the Epo bands corresponding to the duration of incubation with neuraminidase. Protein transfer and Western blotting were employed in preference to chemical staining with silver or Coomassie blue since attempts at the latter revealed that the more desialylated proteins – i.e. those with a higher pI – did not stain, either

Fig. 3.3.1. (Facing page) (a) Epo treated with neuraminidase for increasing lengths of time subjected to isoelectric focusing gel electrophoresis followed by western blot (following protocol in Sections 2.3.6. and 2.3.8.) with anti-Epo antibody. Samples marked \* were selected for further study. (b) The mean pI values of the selected samples were calculated as described in Section 2.3.9.



(b)

Epo sample	Mean pI value
t=0 min	4.21
t=20 min	4.51
t=480 min	5.83
t=1800 min	6.65

because they are unable to stain to the same extent as less desialylated Epo or because they are washed away during the staining procedure. A sugar chain at an N-glycosylation site can contain up to four sialic acids, while at an O-glycosylation site it can have no more than two (see diagram in Fig. 1.3. in Chapter 1). Thus Epo has a theoretical maximum of 14 sialic acid residues per molecule. As expected, 15 bands are visible on the isoelectric focusing gel in Fig. 3.3.1a. (14 bands corresponding to Epo with 1 to 14 sialic acid charges and one band with no charges conferred by sialic acid). Samples incubated with neuraminidase for 0 minutes (Epo sample  $t=0$  min), 20 minutes (Epo sample  $t=20$  min), 480 minutes (Epo sample  $t=480$  min) and 1800 minutes (Epo sample  $t=1800$  min) were chosen for use in bioassays as they represented a broad range of sialylation levels. While it still contained significant amounts of intact Epo, the  $t=20$  min sample also contained bands between pI 5.3 and 6 (possibly corresponding to as many as 5-8 desialylations per molecule). The  $t=480$  min sample exhibited more extensive desialylation, with the presence of some bands corresponding to the lower pI bands in intact Epo, but with the majority of forms displaying anything between 6 and 12 desialylation events per molecule. The  $t=1800$  min sample appeared to be on the verge of complete desialylation. Each sample was assigned a mean pI (Fig. 3.3.1b) by the method described in Section 2.3.9. Mean pI provides a quantitative measure of isoform distribution and was employed here for comparison purposes as it has been reported to be a better predictor of follicle-stimulating hormone (FSH) activity than calculating the proportion of isoforms bands with a pI of less than 5 (Mulders *et al*, 1999)). Epo sample  $t=0$  min, untreated with neuraminidase, had the lowest mean pI (4.21). The mean pI of the samples increased the longer they were incubated in neuraminidase. A 30-hour

incubation in neuraminidase resulted in the sample (t=1800 min) having a mean pI of 6.65.

### **3.3.1.2. In vivo post-hypoxic polycythaemic mouse assay**

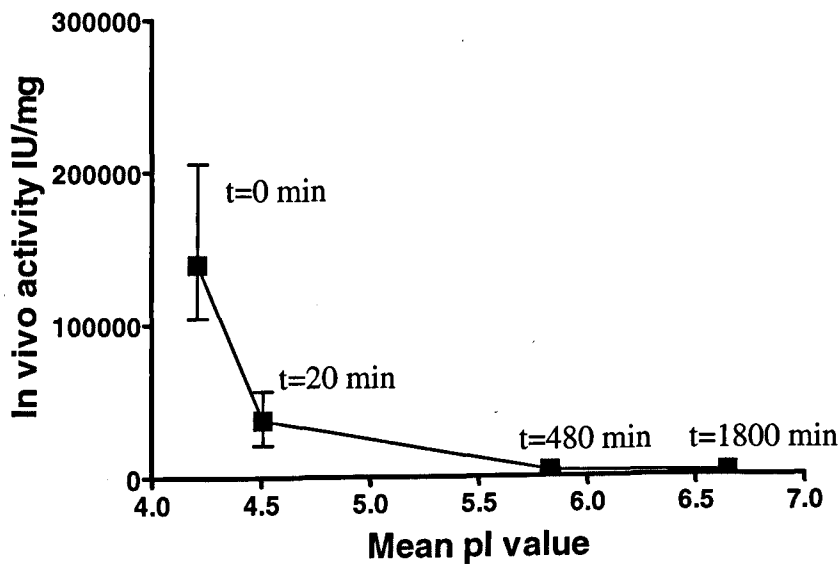
The post-hypoxic polycythaemic mouse assay was carried out on samples t=0 min, 20 min, 480 min and 1800 min (Table 3.3.1.) to ascertain the extent to which desialylation had affected *in vivo* biological activity. The more sensitive polycythaemic assay was chosen for measuring the biological activity of the four samples, the prediction being that the activity of the more extensively desialylated samples would be too low to allow detection by means of the normocythaemic assay. Sample t=0 min was the most biologically active, having an Epo bioactivity of 139,375 IU/mg. An increase in length of treatment with neuraminidase was associated with a progressive decrease in biological activity. The Epo sample treated for the longest period, t=1800 min, exhibited a biological activity of 2,731 IU/mg. This was more than a 50-fold decrease in activity compared with untreated Epo. There is clearly not a linear relationship, however. The loss of activity exceeded 97% after 480 minutes, having reached 73% after 20 minutes, by which time it was very difficult to see any significant desialylation by means of an IEF blot.

### **3.3.1.3. Unmodified AS-E2 bioassay**

AS-E2 is an erythropoietin-dependent human leukemia cell line derived from a patient with acute myeloid leukemia. It expresses a low affinity Epo-R and resembles late BFU-E or CFU-E in that it is responsive to Epo but not IL-3, GM-CSF or stem cell factor (Miyazaki *et al*, 1997).

Table. 3.3.1. Estimates of the specific activities of the untreated and neuraminidase-treated Epo samples, based on *in vivo* post-hypoxic polycythaemic mouse assay in terms of the Second International Standard for Erythropoietin. The figures of the table are plotted in the graph below.

Epo sample	Mean pI value	Epo bioactivity (IU/mg)	95% Confidence interval (IU/mg)
t=0 min	4.21	139,375	104,300-205,684
t=20 min	4.51	37,275	21,121-56,522
t=480 min	5.83	3,948	2,614-5,404
t=1800 min	6.65	2,731	1,960-4,192



The cell-based bioassay used in this project used alamarBlue, a colorimetric reagent which is blue in its oxidized form (resazurin) but which turns pink when reduced, becoming resorufin. Reduction is brought about by the metabolic activity of the cells undergoing measurement as part of the assay.

*In vitro* biological activity of Epo before and after desialylation was determined in the unmodified AS-E2 cell bioassay (see Fig. 3.3.2a). Epo that had not been treated with neuraminidase stimulated a dose-dependent proliferation between 0.03nM and 0.26nM ( $EC_{50} = 0.0614\text{nM}$ ). Epo subjected to 20 minute, 480 minute or 1800 minute neuraminidase treatment showed very similar dose-responses. There was no noticeable change in the slope of the curve or in the maximum proliferation. The  $EC_{50}$  results varied little, all being in the range 0.05833-0.06413nM (Fig. 3.3.2b). The 95% confidence intervals for the estimated  $EC_{50}$  values showed no statistically significant differences between any of the samples.

#### 3.3.1.4. AS-E2/ECL bioassay

*In vitro* biological activity of Epo before and after desialylation was then tested in the modified AS-E2/ECL bioassay (Fig. 3.3.3a) and  $EC_{50}$  values calculated (Fig. 3.3.3b). Epo concentrations were no longer known following the lectin-mediated depletion of Epo containing galactose-exposed sugars. Accordingly, units along the x-axis of Fig. 3.3.3.a represent the concentrations prior to treatment with ECL. Untreated Epo ( $t=0$  min) stimulated proliferation in a dose-dependent manner until maximum proliferation was reached at approximately 0.25nM Epo ( $EC_{50} = 0.04563$  nM). The neuraminidase-treated Epo samples, however, differed from the  $t=0$  min sample to varying degrees. Sample  $t=20$  min reached maximum proliferation at approximately the same concentration but half-maximal response was attained at a slightly higher

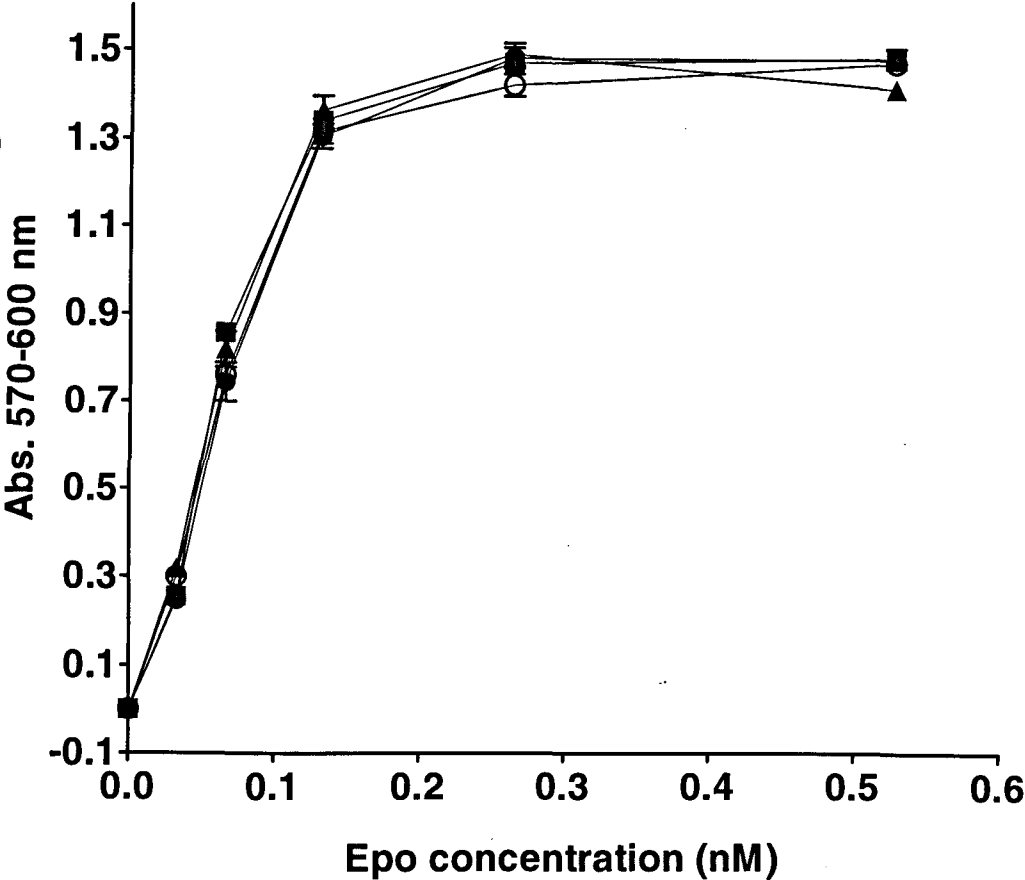
Fig. 3.3.2. (Facing page) Proliferative response of AS-E2 cells to untreated and neuraminidase-treated Epo.

(a) Sample  $t=0$  min (■),  $t=20$  min (▲),  $t=480$  min (●) and  $t=1800$  min (○). Data represent mean  $\pm$  s.e.m. of triplicated samples.

(b)  $EC_{50}$  values and 95% confidence intervals were calculated for the dose-response curves.



(a)

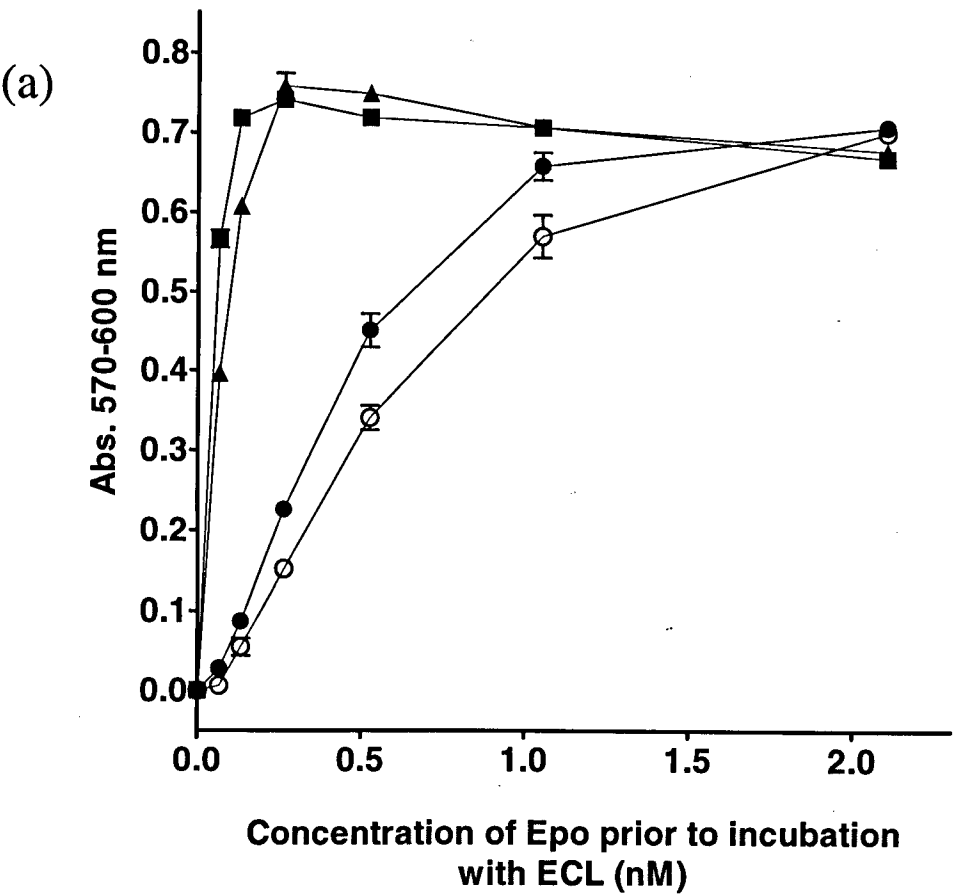


(b)

Epo sample	Best-fit EC <sub>50</sub> values (nM)	95% Confidence interval (nM)
t=0 min (■)	0.05876	0.05733 - 0.06023
t=20 min (▲)	0.05833	0.05446 - 0.06248
t=480 min (●)	0.06413	0.06094 - 0.06747
t=1800 min (○)	0.06140	0.05863 - 0.06431

Fig. 3.3.3. (Facing page) Proliferative response of AS-E2 cells to untreated and neuraminidase-treated Epo subsequent to incubation with agarose-conjugated *Erythrina crista-galli* lectin (ECL). (a) Sample t=0 min (■), t=20 min (▲), t=480 min (●) and t=1800 min (○). Units along the x-axis represent Epo concentrations prior to treatment with ECL. Data represent mean  $\pm$  s.e.m. of triplicated samples.

(b)  $EC_{50}$  values and 95% confidence intervals were calculated for the dose-response curves.



(b)

Epo sample	Best-fit EC <sub>50</sub> values (nM)	95% Confidence Intervals (nM)
t=0 min (■)	0.04563	0.03180 - 0.06547
t=20 min (▲)	0.06405	0.05321 - 0.07709
t=480 min (●)	0.3770	0.3535 - 0.4022
t=1800 min (○)	0.5136	0.4807 - 0.5487

Epo concentration ( $EC_{50} = 0.06405 \text{ nM}$ ). Judging from the confidence intervals (Fig. 3.3.3b), this did not differ significantly from the  $t=0$  min sample, however. By contrast, samples  $t=480$  min and  $1800$  min differed significantly from the untreated sample – the onset of their proliferation being delayed until the concentration had reached at least  $0.06 \text{ nM}$  Epo (at the same concentration, samples  $t=0$  min and  $t=20$  min were at 75% and 50% maximal proliferation respectively), with maximal proliferation being recorded at approximately  $2 \text{ nM}$  Epo. The  $EC_{50}$  was  $0.377 \text{ nM}$  Epo for sample  $t=480$  min and  $0.5136 \text{ nM}$  Epo for sample  $t=1800$  min (the latter indicating a roughly ten-fold decrease in activity compared with untreated Epo). On the basis of the 95% confidence intervals (Fig. 3.3.3b), it is clear that these differ significantly from the  $t=0$  min and  $t=20$  min samples, and from each other.

In order to see whether statistical significance could be obtained between samples  $t=0$  min and  $t=20$  min, the assay was repeated with just these two samples and a dose-response curve with a larger number of lower Epo concentrations (Fig. 3.3.4a). Fig. 3.3.4b shows  $EC_{50}$  values for  $t=0$  min and  $t=20$  min of  $0.02878$  and  $0.04531 \text{ nM}$  respectively. The non-overlapping confidence intervals indicate those results to be statistically significant at the 5% significance level.

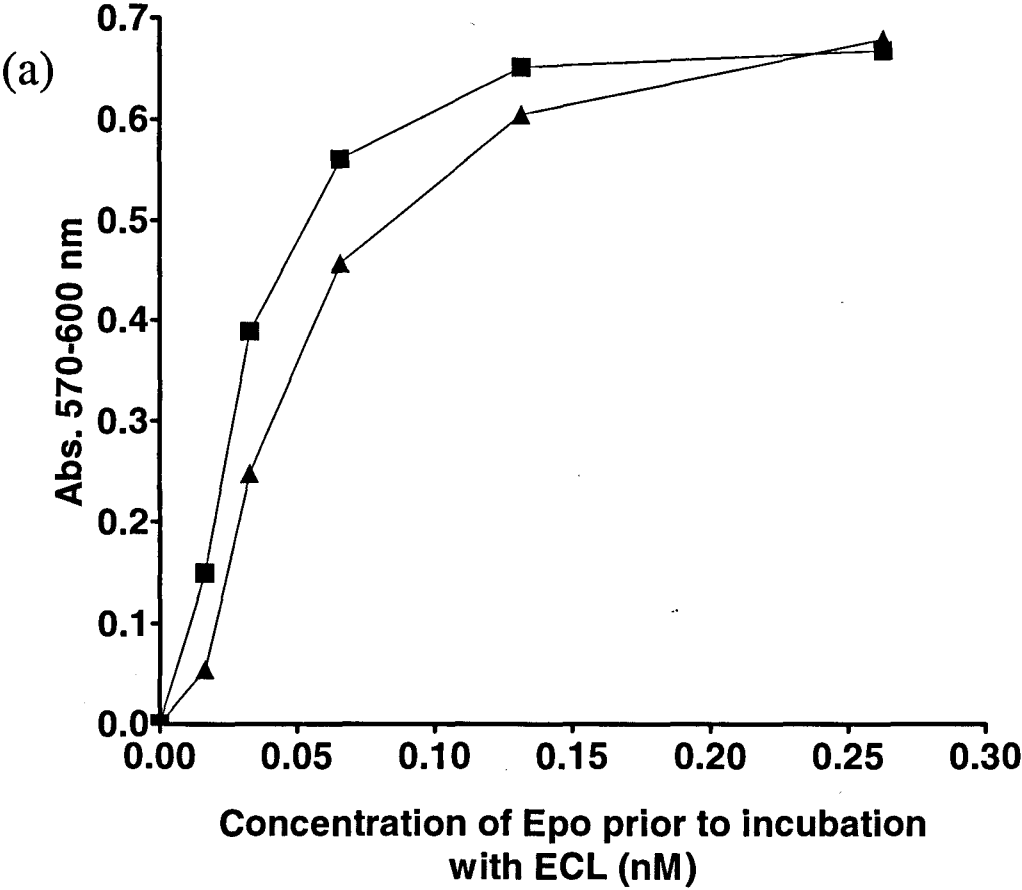
### 3.3.1.5. AS-E2/RCA<sub>120</sub> assay

The same experiment was performed as in Section 3.3.1.4., except that agarose-conjugated ECL was replaced by agarose-conjugated *Ricinus communis* agglutinin (RCA<sub>120</sub>) and the results plotted in Fig. 3.3.5.. Although the results were generally comparable with those obtained as described above, cell death was observed at higher concentrations of Epo, presumably as a result of contamination by the RCA<sub>120</sub> toxin.

Fig. 3.3.4. (Facing page) Proliferative response of AS-E2 cells to untreated and 20 minute-neuraminidase-treated Epo subsequent to incubation with agarose-conjugated ECL; differentiation between samples  $t=0$  min and  $t=20$  min.

(a) Sample  $t=0$  min (■) and  $t=20$  min (▲). Units along the x-axis represent the concentrations prior to treatment with ECL. Data represent mean  $\pm$  s.e.m. of triplicated samples (where an error bar is not shown, it lies within the dimensions of the symbol).

(b)  $EC_{50}$  values and 95% confidence intervals were calculated for the dose-response curves. Data are representative of two independent experiments.



(b)

Epo sample	Best-fit $EC_{50}$ values (nM)	95% Confidence Intervals (nM)
t=0 min (■)	0.02878	0.02801 - 0.02958
t=20 min (▲)	0.04531	0.04323 - 0.04748

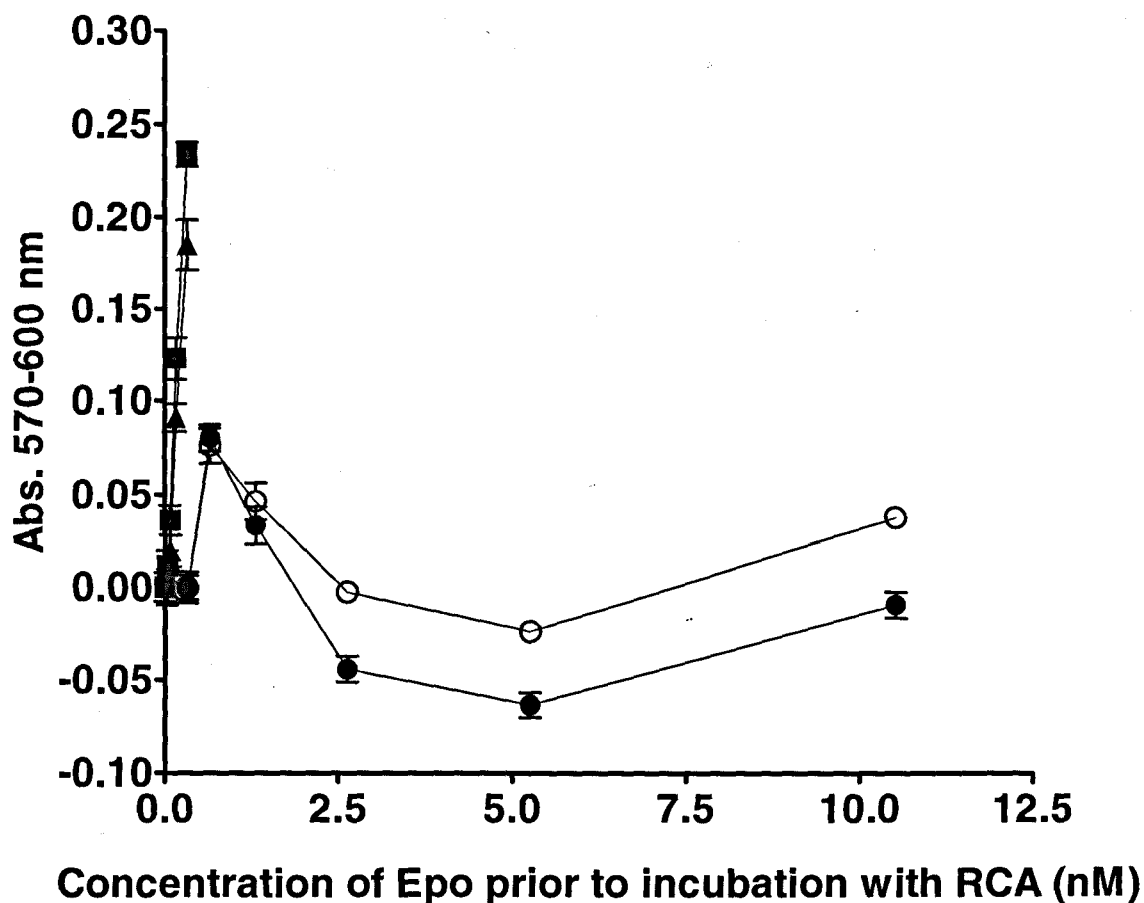


Fig. 3.3.5. Proliferative response of AS-E2 cells to untreated and neuraminidase-treated Epo subsequent to incubation with agarose-conjugated RCA<sub>120</sub>; replacement of agarose-conjugated ECL with agarose-conjugated *Ricinus communis* agglutinin (RCA<sub>120</sub>).

(a) Sample t=0 min (■), t=20 min (▲), t=480 min (●) and t=1800 min (○).

Units along the x-axis represent the concentrations prior to treatment with RCA<sub>120</sub>. Data represent mean  $\pm$  s.e.m. of triplicated samples.

(b) EC<sub>50</sub> values and 95% confidence intervals were calculated for the dose-response curves.

### 3.3.1.6. Depletion of Epo was lectin-dependent

To confirm that the depletion of Epo in these assays was not due to adhesion to agarose or degradation during the six-hour incubation, the four Epo samples were incubated with agarose alone and then tested in the AS-E2 bioassay. Fig. 3.3.6. shows that the four Epo samples incubated for six hours with agarose alone did not exhibit the same trend as when they were incubated with lectin-bound agarose, in that Epo treated with neuraminidase for eight hours or more ( $t=480$  min and  $t=1800$  min) did not give a lower proliferative response.

The most extensively desialylated Epo sample ( $t=1800$  min) incubated with agarose-conjugated ECL in the presence or absence of lactose at an eluting concentration of 200 mM was assayed using AS-E2 cells (Fig. 3.3.7.). Epo sample  $t=1800$  min incubated in the absence of lactose had an  $EC_{50}$  value of 0.65 nM, similar to that obtained in Fig. 3.3.3. for the same sample and type of assay (i.e. AS-E2/ECL assay), whereas the same  $t=1800$  min sample incubated with ECL in the presence of lactose had a much lower  $EC_{50}$  value in the region of 0.1 nM, similar to the  $EC_{50}$  value obtained in the unmodified AS-E2 (Fig. 3.3.2.). Lactose contains galactose and therefore is recommended for eluting ligands from ECL. This experiment demonstrates that the sugar specific for inhibiting binding to ECL abolishes the effect of depleting desialylated Epo described in Section 3.3.1.4.

### 3.3.1.7. Lectin free in solution does not result in depletion of Epo

ECL (0.375mg/ml – corresponding to 2.5x the concentration of ECL used in the agarose-conjugated form of the assays) was added free in solution to the AS-E2 bioassays measuring the potency of the  $t=0$  min and  $t=1800$  min Epo samples. This was done to see whether it was possible to distinguish between intact and desialylated



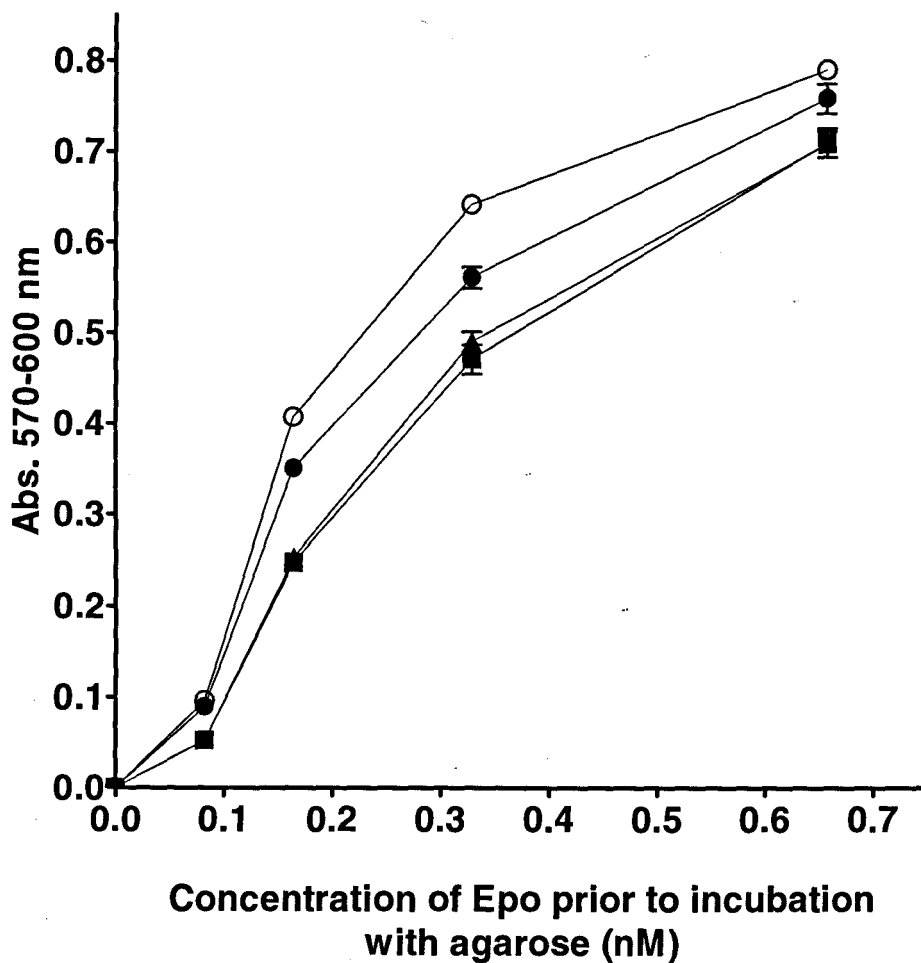


Fig. 3.3.6. Proliferative response of AS-E2 cells to untreated and neuraminidase-treated Epo subsequent to incubation with agarose alone. Sample t=0 min (■), t=20 min (▲), t=480 min (●) and t=1800 min (○). Units along the x-axis represent the concentrations prior to treatment with agarose. Data represent mean  $\pm$  s.e.m. of triplicated samples (where an error bar is not shown, it lies within the dimensions of the symbol).

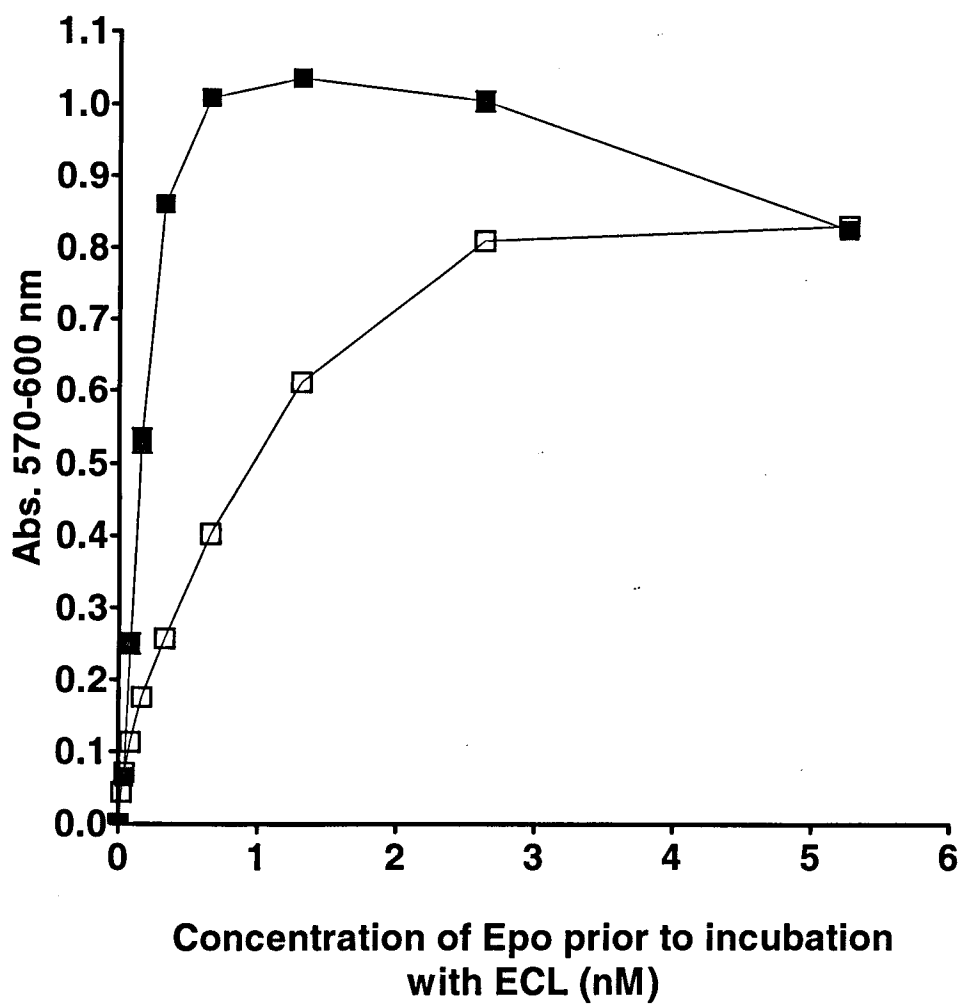


Fig. 3.3.7. Proliferative response of AS-E2 cells to neuraminidase-treated Epo (sample t=1800 min) subsequent to incubation with agarose-conjugated ECL with (■) or without (□) the presence of lactose (200 mM). Units along the x-axis represent the concentrations prior to treatment with ECL. Data represent mean  $\pm$  s.e.m. of triplicated samples (where an error bar is not shown, it lies within the dimensions of the symbol).

Epo without any need for pre-incubation with the agarose-bound form of the lectin.

Fig. 3.3.8. shows that the free ECL did not reduce the proliferation of AS-E2 cells stimulated with the  $t=1800$  min Epo sample. In the unmodified AS-E2 bioassay, Epo sample  $t=1800$  min is 1.067 times as active as  $t=0$  min. When ECL is added free to the medium of the assay at a concentration of 0.375mg/ml, Epo sample  $t=1800$  min still has a higher potency than  $t=0$  min (1.33 times as potent). ECL free in solution did not competitively inhibit Epo containing a higher proportion of terminal galactose residues from binding the Epo receptor and stimulating proliferation.

### **3.3.1.8. Comparison of the AS-E2, AS-E2/ECL and the in vivo mouse bioassays**

The percentage activity of the desialylated samples was calculated relative to untreated Epo ( $t=0$  min) for each of the three types of assay (Fig. 3.3.9a). The results were plotted against their mean pI values (Fig. 3.3.9b).

The pI of intact Epo (Epo sample  $t=0$  min,  $pI=4.21$ ) was only 0.3 pI units lower than slightly desialylated Epo (Epo sample  $t=20$  min,  $pI=4.51$ ) indicating a slightly higher level of sialylation, but this corresponded to a 73% drop in activity. Epo samples  $t=480$  min and  $t=1800$  min were desialylated to a greater extent (indicated by mean pI values of 5.83 and 6.65 respectively) and had lost nearly all potency, being well below 5% initial starting activity.

Samples tested in the unmodified AS-E2 bioassay displayed the same level of activity relative to that of untreated Epo (approximately 100%), irrespective of sialylation level.

In the AS-E2/ECL bioassay, the slightly desialylated Epo sample  $t=20$  min exhibited approximately 70% of the activity level of Epo untreated with neuraminidase, whereas the two extensively desialylated Epo samples ( $t=480$  min and  $t=1800$  min)

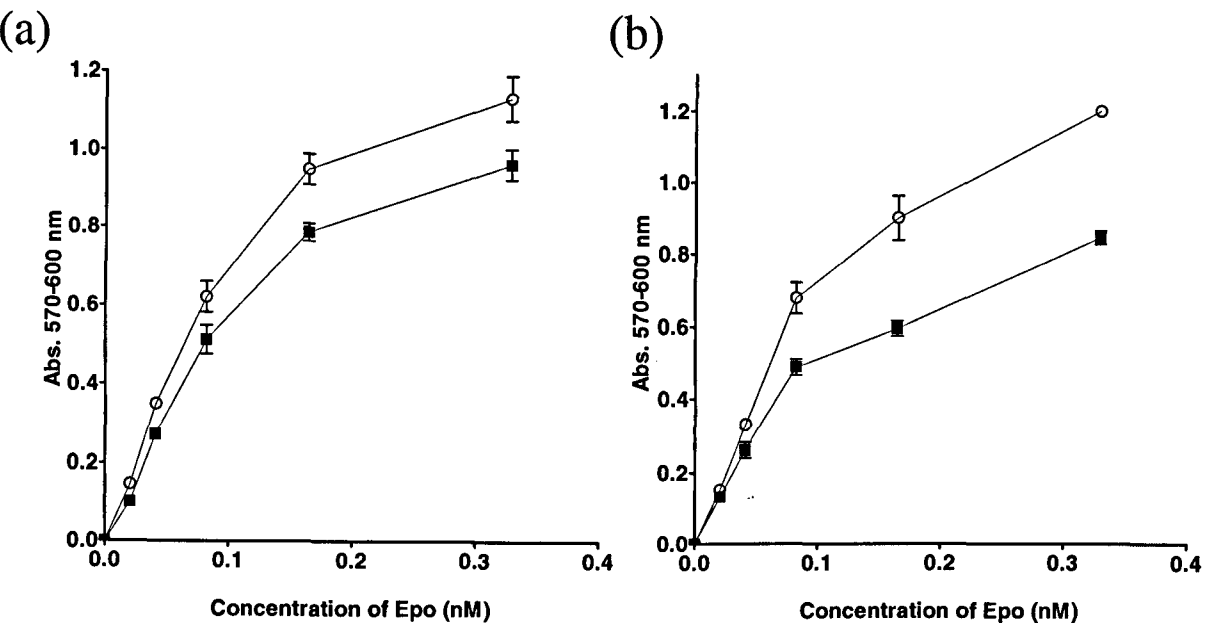


Fig. 3.3.8. Effect of *Erythrina crista-galli* lectin free in solution.

- (a) Cells were grown in normal assay medium supplemented with sample t=0 min Epo (■) and sample t=1800 min Epo (○).
- (b) ECL (at a concentration of 0.375 mg/ml) was included in the assay medium. Sample t=0 min (■) and t=1800 min (○). Data represent mean +/- s.e.m. of triplicated samples.

Epo sample	Best-fit EC <sub>50</sub> values (nM) for normal assay	Best-fit EC <sub>50</sub> values (nM) for assay in which free ECL was included in the medium
t=0 min (■)	0.09715 (95% confidence intervals= 0.07812 – 0.1208)	0.1307 (95% confidence intervals= 0.05989 – 0.2854)
t=1800 min (○)	0.09101 (95% confidence intervals = 0.07312 – 0.1133)	0.09815 (95% confidence intervals= 0.06618 – 0.1456)

Fig. 3.3.9. Comparison of *in vivo*, AS-E2 and AS-E2/ECL bioassays.

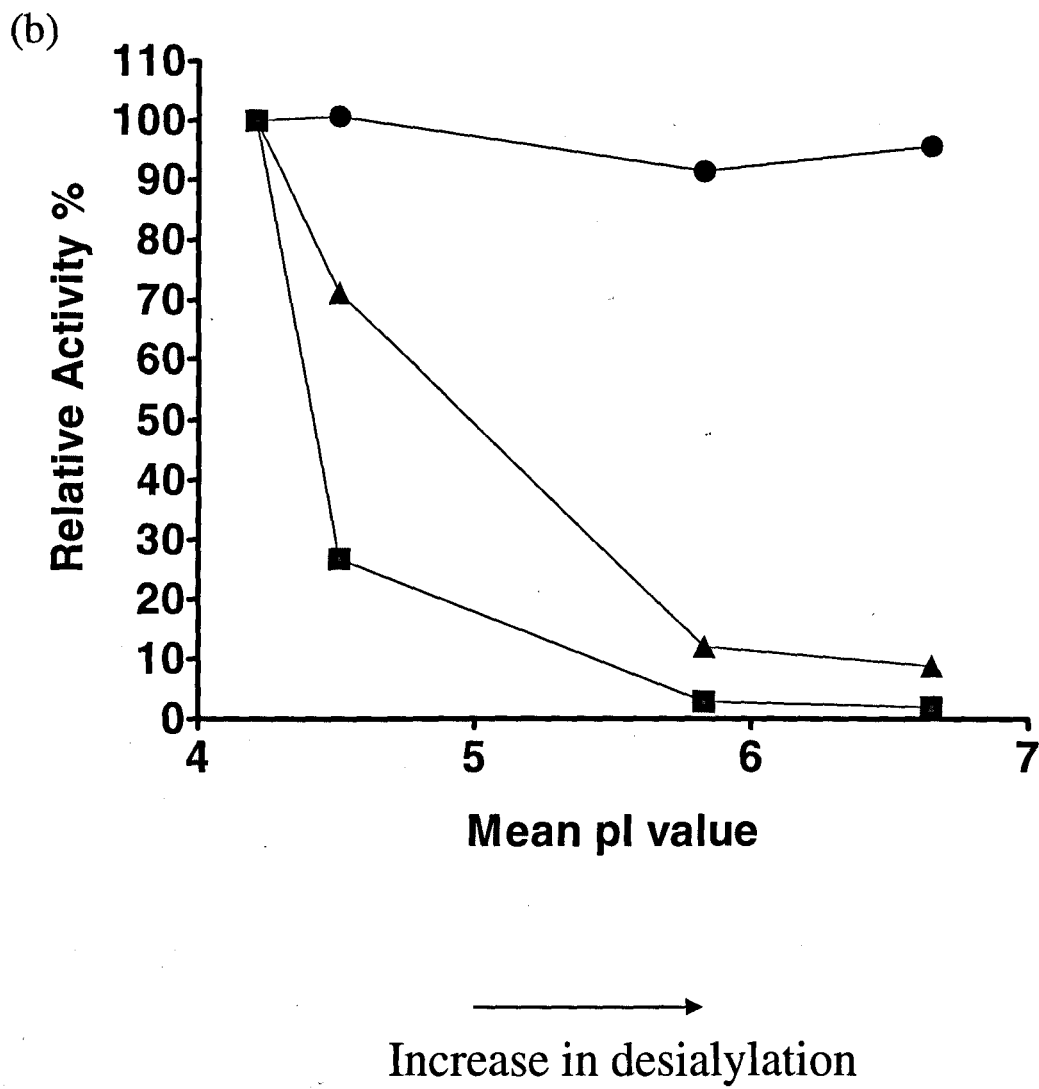
(a) Percentage activity of desialylated samples relative to untreated Epo for each of the three assays.

(b) (Facing page) Relative percentage activity plotted against mean pI value.

*In vivo* bioassay (■), AS-E2 bioassay (●), AS-E2/ECL bioassay (▲).

(a)

Epo sample	Mean pI value	<i>In vivo</i> % activity	AS-E2 % activity	AS-E2/ECL % activity
t=0 min	4.21	100	100	100
t=20 min	4.51	26.74	100.7	71.23
t=480 min	5.83	2.85	91.6	12.10
t=1800 min	6.65	1.96	95.7	8.89



were found to be only about 10% as active. Results obtained using the AS-E2/ECL bioassay were similar to those obtained with the *in vivo* bioassay (Pearson correlation  $R=0.905$ ,  $P=0.095$ ). In essence, lower levels of sialylation, as indicated by lower mean pI values, correlated with a decrease in activity, albeit with less sensitivity (particularly in the slightly desialylated range).

### **3.3.2. Sample set 2: Range of pharmaceutical Epo preparations generated by different production and purification procedures**

#### **3.3.2.1. Background information**

In addition to the experimentally desialylated Epo samples, a second set of samples comprising a range of pharmaceutical Epo preparations was tested using the AS-E2/ECL bioassay. The pharmaceutical Epo preparations, which were kindly donated by Dr Yuen, NIBSC, and described in reference (Yuen *et al*, 2003), were produced by Chinese Hamster Ovary (CHO) cells but were cultured and purified differently in order to effect a variation in their glycan structures. Pharmaceutical Epo preparations Gr and L2 were made in roller bottles with Dulbecco's-modified Eagle's medium (DMEM) as the culture medium, while pharmaceutical Epo preparations M2G and SpB were made in a hollow fibre bioreactor with DMEM/Nutrient Mixture F12 Ham's medium containing 5% FCS (v/v). Purification was the same for all the samples except that sample SpB was passed over a cation-exchange chromatography column so as to concentrate more basic Epo molecules.

Glycan mapping has previously been carried out on the Epo preparations of sample set 2 and their *in vivo* potencies measured to enable the relationships between carbohydrate structure and biological activity to be studied (Yuen *et al*, 2003). A

direct correlation was observed between tetra-sialylated tetra-antennary structure content and *in vivo* biological activity (Pearson correlation  $R = 0.974$ ,  $P < 0.0005$ ), with sample SpB having the lowest proportion of tetra-sialylated tetra-antennary sugars and the lowest *in vivo* activity (yet the highest *in vitro* activity). No relationship was found between *in vivo* potency and the proportion of the trisialylated tri-antennary-enriched Epo, however (Pearson correlation  $R = -0.505$ ,  $P > 0.05$ ).

Fig. 3.3.10. is the IEF gel from reference (Yuen *et al*, 2003) which was kindly loaned, for densitometric analysis purposes, to calculate the mean pI values listed in Table 3.3.2. The values range from 4.962 for the most basic SpB sample to 4.127 for the most acidic L2 sample (the order of increase in acidity being SpB<M2G<Gr<L2). The Yuen *et al* reference (2003) provided post-hypoxic polycythaemic mouse assay results and these are reproduced here by kind permission (Table. 3.3.3.). Potencies ranged from 28,300 IU/mg for SpB the least active pharmaceutical Epo preparation (it was also the least acidic if mean pI value is used as a guide), to 94,600 IU/mg for L2, the most biologically active and acidic sample. Biological activity was very closely related to mean pI value (Pearson correlation  $R = -0.976$ ,  $P < 0.05$ ), with the order in which samples increased in activity mirroring exactly the order in which their mean pI values decreased.

### 3.3.2.2. Unmodified AS-E2 bioassay

When tested in the unmodified AS-E2 assay (Section 2.2.4.), the four pharmaceutical Epo preparations gave very similar potencies in terms of  $EC_{50}$  values, which were all in the range 0.06287-0.07158 nM (see Fig. 3.3.11.).



Fig. 3.3.10. (Facing page) Isoelectric focusing of the four pharmaceutical Epo preparations detected by Western blotting: Gr, SpB, M2G and L2 (kindly donated for the purposes of densitometric analysis and reproduced here with permission from Yuen *et al* (2003)).

Table 3.3.2. Calculation of the mean pI values of the four pharmaceutical Epo preparations (see Section 2.3.9. for procedure followed).

Pharmaceutical Epo preparation	Mean pI value
Gr	4.28
SpB	4.96
L2	4.13
M2G	4.40

Table 3.1.3. Isoelectric points of the specific subunits of the four

pharmacological type isozymes, based on in vitro studies

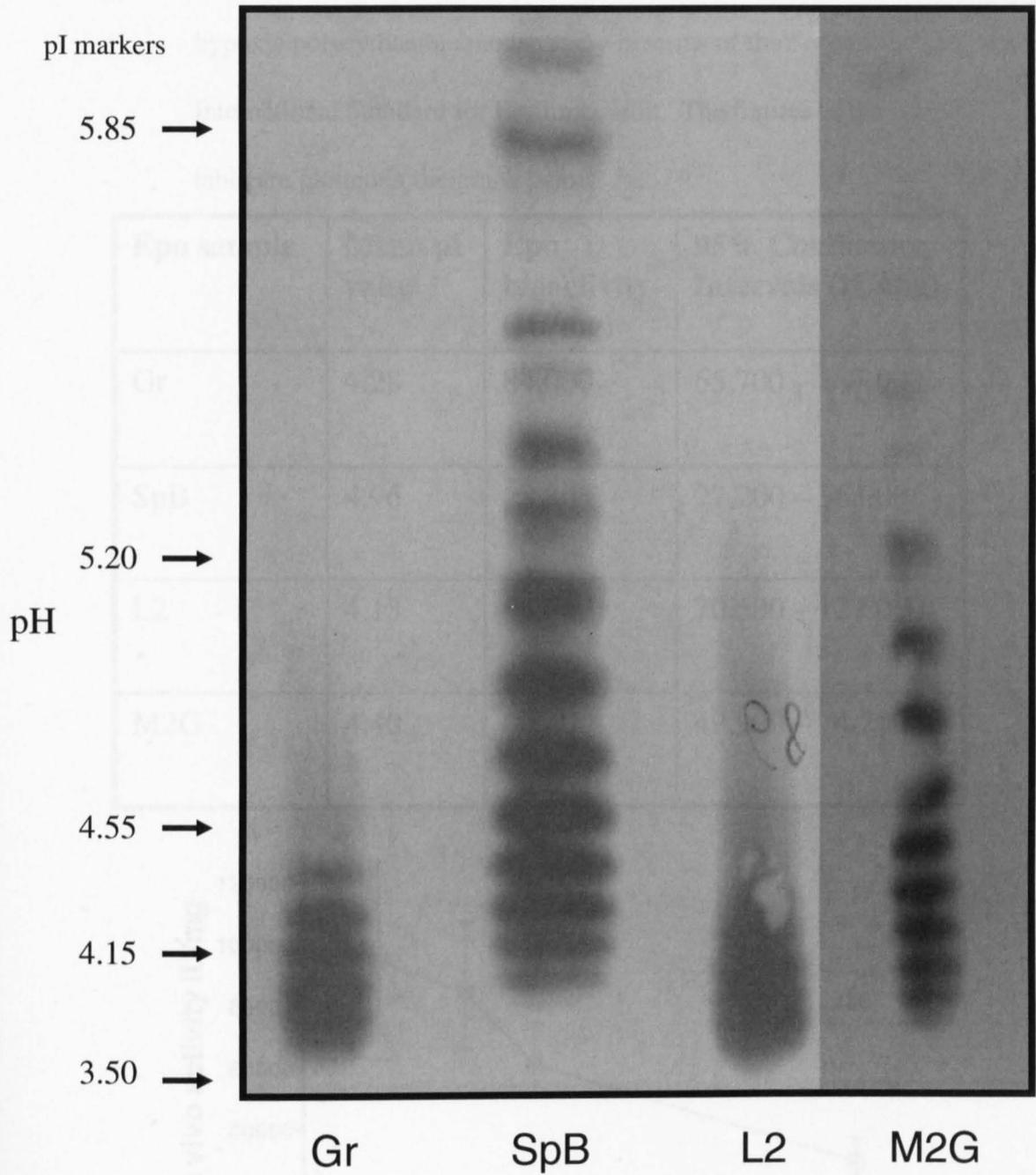


Table. 3.3.3. Estimates of the specific activities of the four pharmaceutical Epo preparations, based on *in vivo* post-hypoxic polycythaemic mouse assay in terms of the Second International Standard for Erythropoietin. The figures of the table are plotted in the graph below.

Epo sample	Mean pI value	Epo bioactivity (IU/mg)	95% Confidence Intervals (IU/mg)
Gr	4.28	84,000	65,700 – 107,000
SpB	4.96	28,300	22,200 – 36,000
L2	4.13	94,600	70,500 – 127,000
M2G	4.40	60,800	49,900 – 74,200

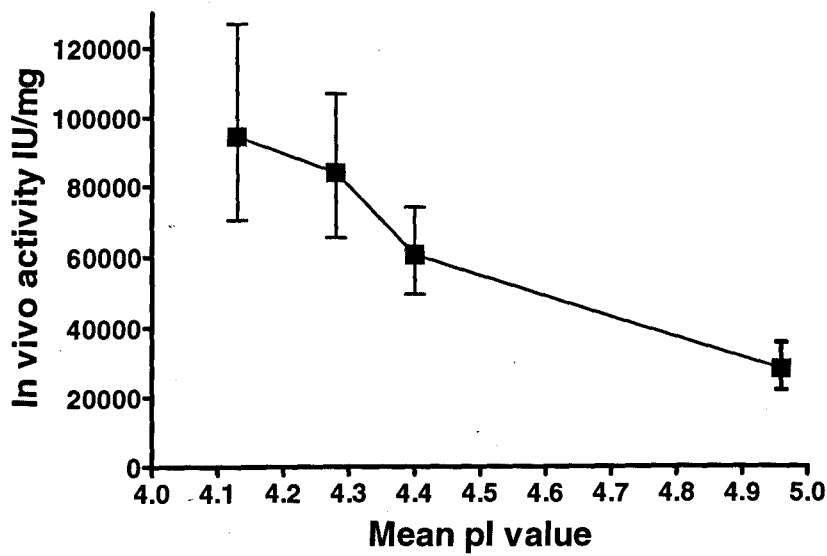
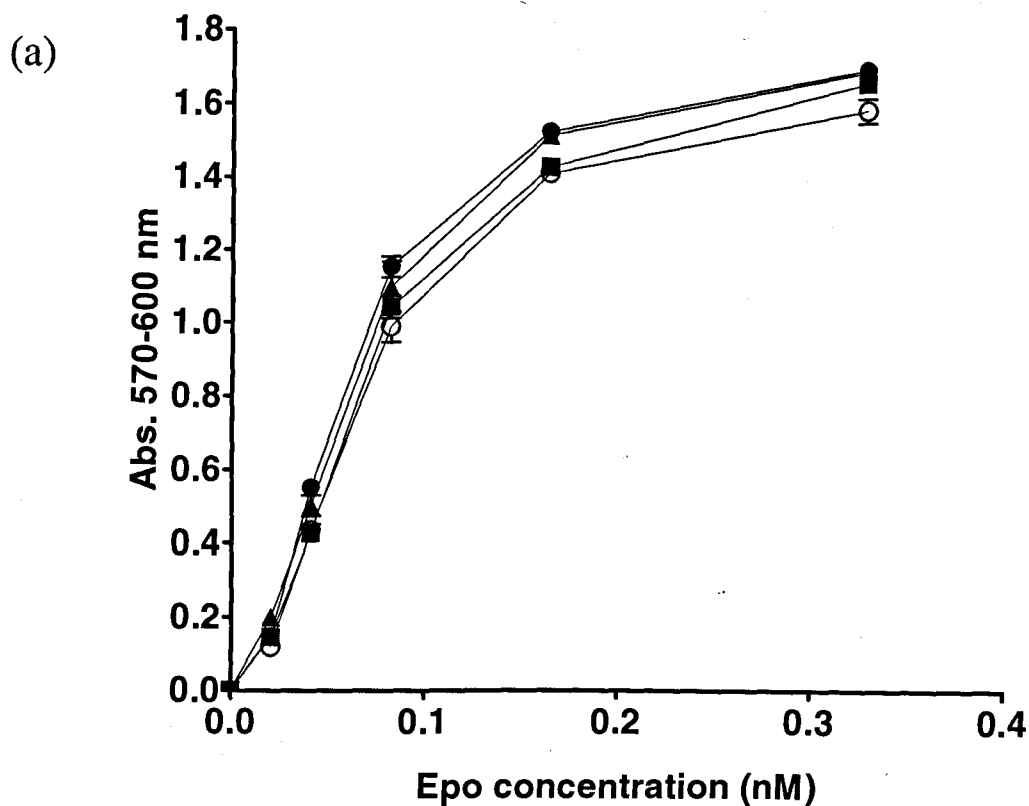


Fig. 3.3.11. (Facing page) Proliferative response of AS-E2 cells to the four pharmaceutical Epo preparations.

(a) Sample Gr (■), SpB (▲), L2 (●) and M2G (○).

Data represent mean  $\pm$  s.e.m. of triplicated samples.

(b)  $EC_{50}$  values and 95% confidence intervals were calculated for the dose-response curves.



(b)

Epo sample	Best-fit $EC_{50}$ value (nM)	95% Confidence interval (nM)
Gr (■)	0.07158	0.06639 - 0.07717
SpB (▲)	0.06769	0.06156 - 0.07444
L2 (●)	0.06287	0.05904 - 0.06695
M2G (○)	0.07093	0.06555 - 0.07675

### 3.3.2.3. AS-E2/ECL bioassay

The AS-E2/ECL bioassay, in contrast, differentiated between the four samples, in that their  $EC_{50}$ -indicated potencies rose in the following order: SpB<M2G<Gr<L2 (see Fig. 3.3.12.). The differences between any two pairs of samples were statistically significant at the 5% level ( $P<0.05$ ), their  $EC_{50}$  values ranging from 0.07998 for L2 (indicating the highest level of activity) to 0.1254 for SpB (indicating the lowest level of activity). This was verified further by statistical analysis using RANDOM, an in-house program which calculates relative potencies. The  $EC_{50}$  values of samples L2 and Gr were very close, however, and did not differ more markedly from each other than in the unmodified AS-E2 bioassay.

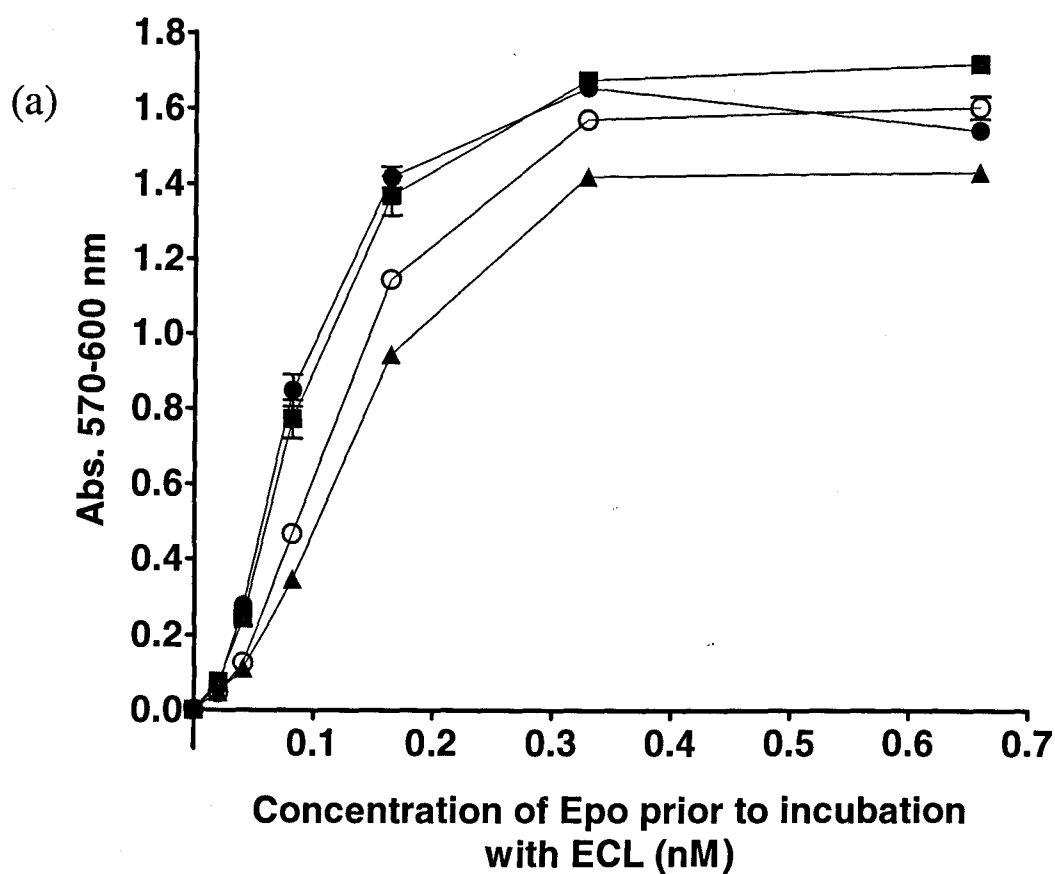
### 3.3.2.4. Comparison of the AS-E2, AS-E2/ECL, in vivo mouse and IEF assay

Post-hypoxic polycythaemic mouse assay results (Table. 3.3.3.) followed the same pattern of increasing biological activity as was obtained in the AS-E2/ECL assay (i.e. SpB<M2G<Gr<L2). Mean pI values, on the other hand, decreased, presumably indicating an increase in the proportion of sialic acids (see Table. 3.3.2.). Potencies relative to L2 assigned by *in vivo* assay, unmodified AS-E2 assay and AS-E2/ECL bioassay are set out in Fig. 3.3.13a. and plotted against mean pI values in Fig. 3.3.13b. *In vivo* activity and AS-E2/ECL activity directly correlate (Pearson correlation  $R=0.914$ ,  $P=0.086$ ), both decreasing as the mean pI values increase. In contrast, AS-E2 activity remains near the 100% level, regardless of mean pI value. As was observed in the case of the artificially desialylated Epo samples in Section 3.3.1., the AS-E2/ECL bioassay was ultimately not as sensitive as the *in vivo* assay, with sample SpB reaching around 58% of the activity of L2, compared with 30% for the mouse assay. Somewhat surprisingly, Gr activity appeared to be lower relative to L2 when

Fig. 3.3.12. (Facing page) Proliferative response of AS-E2 cells to the four pharmaceutical Epo preparations subsequent to incubation with agarose-conjugated ECL.

(a) Sample Gr (■), SpB (▲), L2 (●) and M2G (○). Units along the x-axis represent the concentrations prior to treatment with agarose. Data represent mean  $\pm$  s.e.m. of triplicated samples (where an error bar is not shown, it lies within the dimensions of the symbol).

(b)  $EC_{50}$  values and 95% confidence intervals were calculated for the dose-response curves. Data are representative of three independent experiments.



(b)

Epo sample	Best-fit $EC_{50}$ value (nM)	95% Confidence interval (nM)
Gr (■)	0.09001	0.08579 - 0.09445
SpB (▲)	0.1254	0.1193 - 0.1318
L2 (●)	0.07998	0.07582 - 0.08436
M2G (○)	0.1140	0.1094 - 0.1188



Fig. 3.3.13. Comparison of *in vivo*, AS-E2 and AS-E2/ECL bioassays for the four pharmaceutical Epo preparations.

(a) Relative percentage activity for the three types of assay, taking L2 as the reference sample.

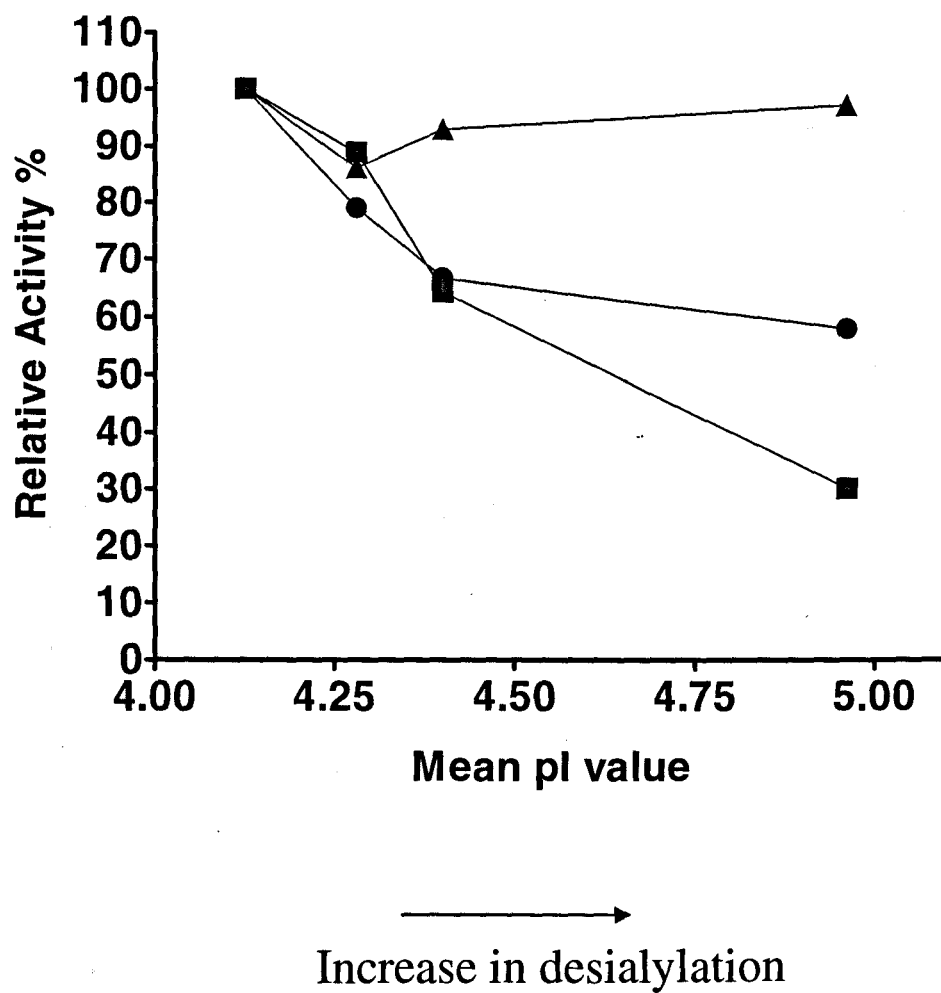
(b) (Facing page) Relative percentage activity plotted against mean pI value.

*In vivo* bioassay (■), AS-E2/ECL bioassay (●), AS-E2 bioassay (▲).

(a)

Epo sample	Mean pI value	<i>In vivo</i> activity %	AS-E2 activity %	AS-E2/ECL activity %
L2	4.127	100	100	100
SpB	4.962	30.0	97.2	57.9
M2G	4.401	64.3	92.9	66.6
Gr	4.281	88.8	86.0	78.9

(b)



detected by the AS-E2/ECL assay compared to when tested in the *in vivo* assay. This can, however, be explained by the fact that Gr was also less active in the unmodified AS-E2 assay.

### **3.3.3. Sample set 3: pharmaceutical Epo fractionated by anion-exchange HPLC**

#### **3.3.3.1. Background on the anion-exchange fractionated samples**

To generate, from a single batch of Epo, a range of samples with varying ratios of acidic to basic isoforms, pharmaceutical-grade Epo (kindly donated by Amgen, USA) was fractionated by semi-preparative anion-exchange HPLC using a MonoQ column (as described in Section 2.3.11.). Fig. 3.3.14. shows a plot of the elution profile (0.025-1 M NaCl gradient). The preparative scale ion-exchange chromatography of Epo used in these studies produced an elution pattern corresponding to that of the known isoform distribution by IEF, although without baseline resolution. Fractions eluted from the column were then tested by various means; isoelectric focusing gel electrophoresis, glycoanalysis, two *in vivo* methods, and the AS-E2 and AS-E2/ECL *in vitro* bioassays.

#### **3.3.3.2. Isoelectric focusing**

Fractions (0.8 ml) were collected as they eluted from the MonoQ column and run on a pH 2.5-7 IEF gradient (Section 2.3.6.). The IEF gel of Fig. 3.3.15. shows fractions 21 to 42 - corresponding to minutes 24.4 to 32.8 min on the plot of the elution profile (Fig. 3.3.14.) - with successive fractions having been pooled. Fractions 21 to 24, 27 to 30, 33 to 36, and 39 to 42 were further pooled and designated as Epo Fractions A, B, C and D respectively.

Fig. 3.3.14. HPLC UV trace of pharmaceutical-grade Epo (Amgen) eluted from an anionic exchange column (MonoQ). The turquoise slope represents the ramp set to run from 0% to 20% 1M NaCl. The dark blue curve tracks the absorbance of the eluate at 276 nm as a percentage of maximum. See Section 2.3.11. for further details.

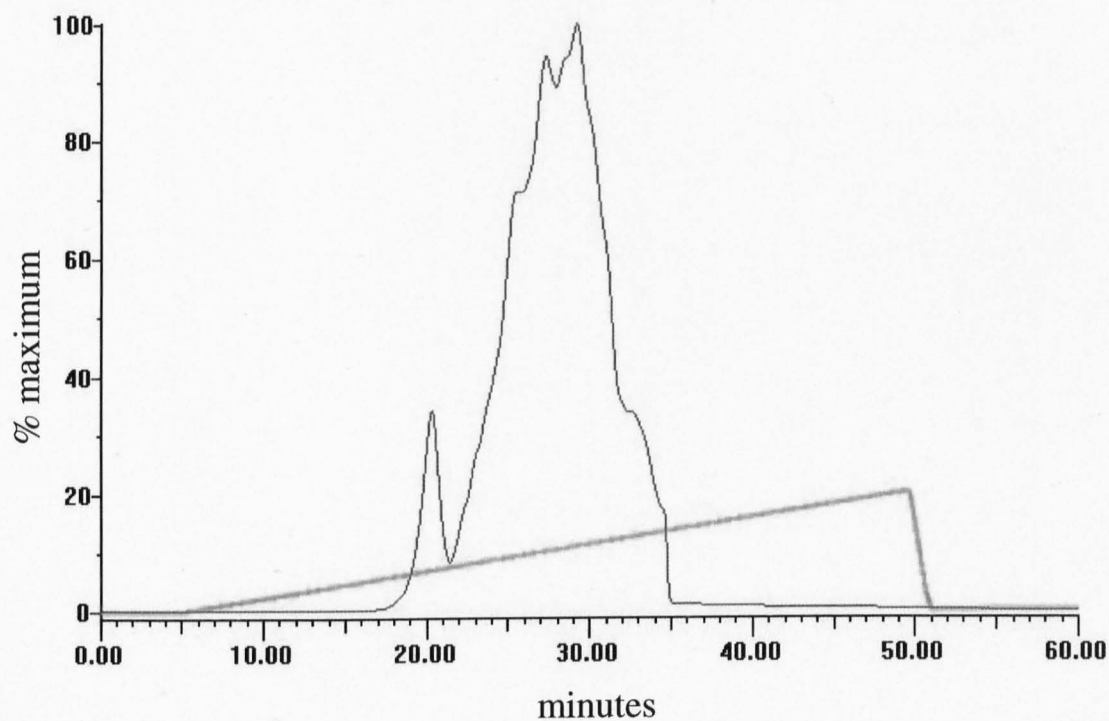
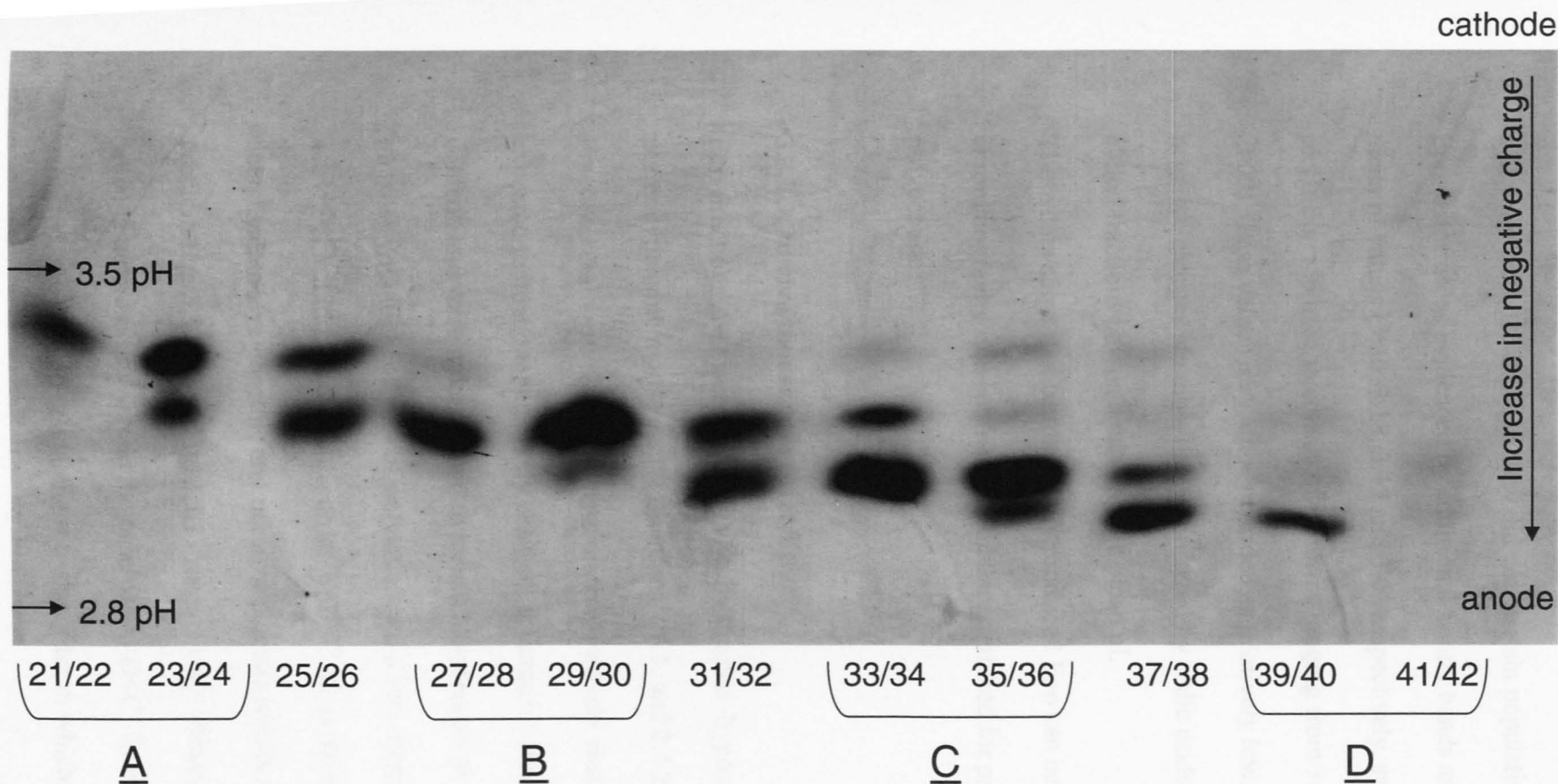


Fig. 3.3.15. (Facing page) Isoelectric focusing gel of ion-exchange HPLC-separated fractions of a batch of pharmaceutical-grade Epo (Amgen).

Table 3.3.4. Table of mean pI values and pI values of the strongest band of the ion-exchange HPLC-separated Epo Fractions on the facing page.

<b>Fraction</b>	<b>Mean pI value</b>	<b>pI of strongest band</b>
A	3.30	3.32
B	3.18	3.18
C	3.13	3.12
D	3.06	3.03



Key:

A, B, C and D= samples submitted for Glycan mapping and *in vivo* and *in vitro* testing x/y (e.g. 21/22)= fractions x and y pooled (0.8 ml fractions collected).

Epo Fraction A was enriched in Epo with a pI value of 3.32, Fraction B in Epo with a pI value of 3.18, Fraction C in Epo with a pI value of 3.12 and Fraction D in Epo with a pI value of 3.03, although they did not contain populations composed solely of these Epo isoforms, as evidenced by additional fainter bands and slight differences in their mean pI values (3.30, 3.18, 3.13 and 3.06 respectively, see Table 3.3.4.). With recHuEpo reported to consist of isoforms ranging from 3.5-4.5 pI units (Schlags *et al*, 2002), these values may all be regarded as relatively low. If it is accepted that Epo's negative charges are most likely conferred by sialic acids, this is consistent with later eluted fractions having a more acidic mean pI.

These data show that commercially produced Epo can be separated by anion exchange chromatography into subfractions that are enriched for particular isoforms of the polypeptide.

### 3.3.3.3. In vivo mouse assays for Epo

Epo Fractions A-D were tested *in vivo* by the post-hypoxic polycythaemic and normocythaemic mouse assays (Sections 2.4.1. and 2.4.2. respectively). Epo Fractions that were retained longer on the positively charged MonoQ column were expected to be more negatively charged, presumably due to a greater sialic acid content, and were indeed found to have a lower mean pI value. Biological potencies increase with higher levels of sialylation and it was therefore expected that *in vivo* activities would increase in the order A<B<C<D as the corresponding mean pI values decreased. Polycythaemic and normocythaemic results are depicted in a table and a graph in Fig. 3.3.16. and Fig. 3.3.17 respectively. Biological activities obtained in the polycythaemic assay were in the order B<A<D<C. This order did not match the order expected, showing no resemblance to the order in which Epo Fractions were eluted

Fig. 3.3.16. (Facing page) (a) Table containing estimates of the specific activities of the four ion-exchange fractionated Epo samples by *in vivo* post-hypoxic polycythaemic mouse assay, in terms of the Second International Standard for Erythropoietin. (b) Graph of the *in vivo* potencies (including 95% confidence intervals) of Epo Fractions A, B, C and D (A-D corresponding to the order in which the samples came off the anionic exchange column, A first), plotted against mean pI value.



(a)

Epo Fraction	Mean pI value	Epo bioactivity (IU/mg)	95% Confidence Intervals (IU/mg)
Starting material	-	161,001	128,053-202,432
A	3.30	184,774	137,689-247,965
B	3.18	162,039	134,382-195,389
C	3.13	229,848	157,688-335,127
D	3.06	196,743	164,055-235,950

(b)

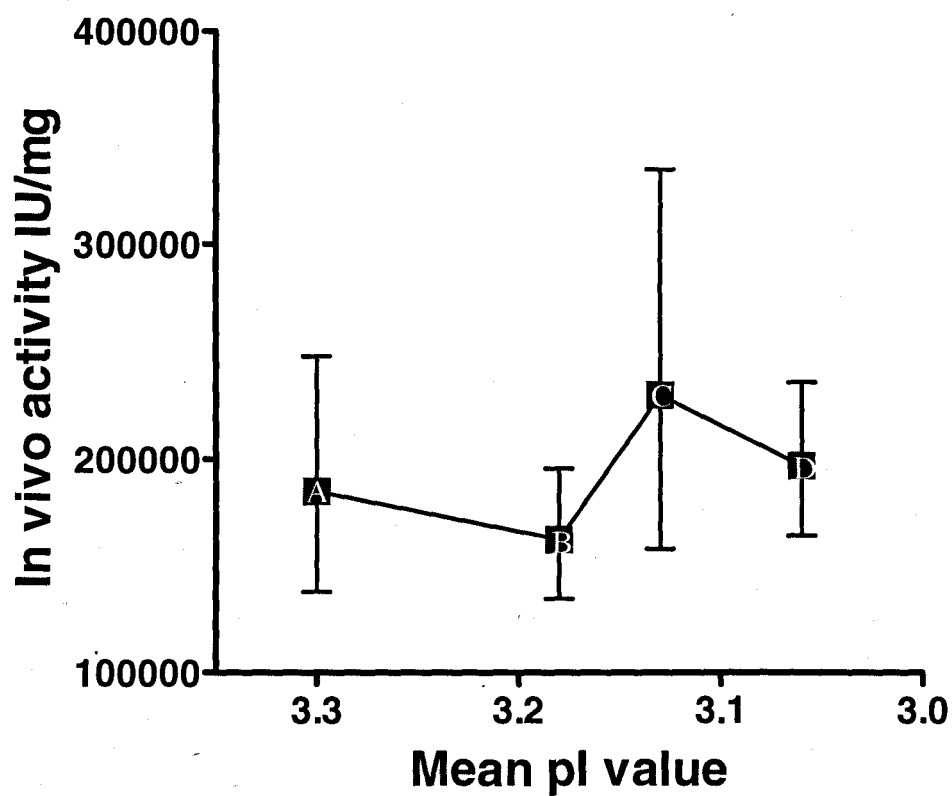
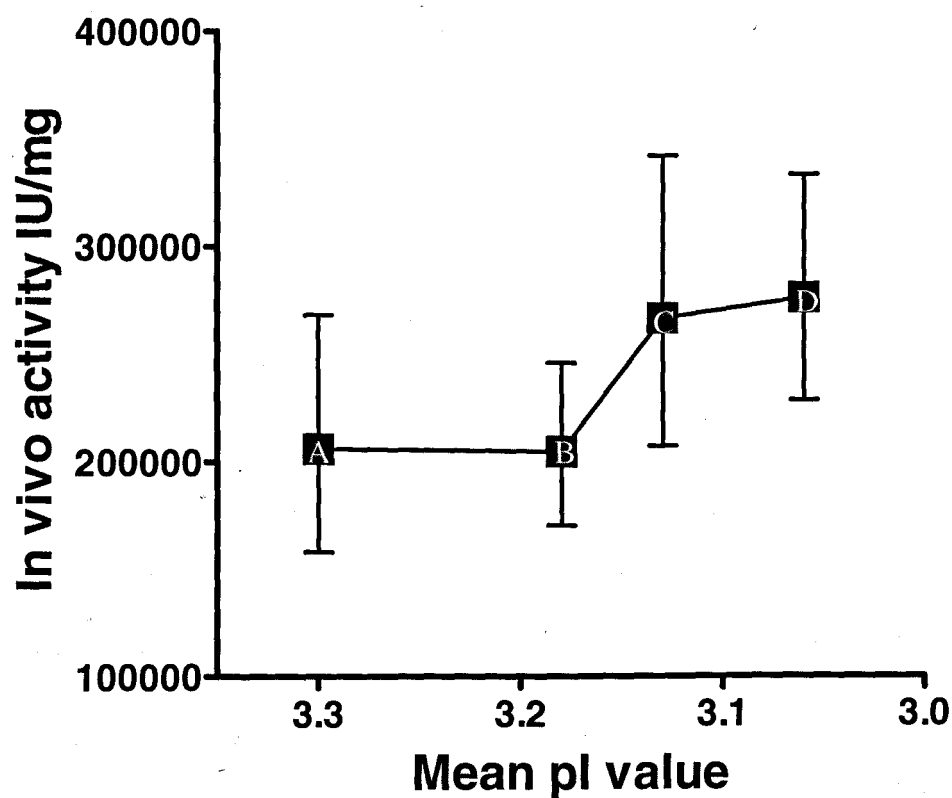


Fig. 3.3.17. (Facing page) (a) Table containing estimates of the specific activities of the four ion exchange fractionated Epo samples by *in vivo* normocythaemic mouse assay, in terms of the Second International Standard for Erythropoietin. (b) Graph of the *in vivo* potencies (including 95% confidence intervals) of Epo Fractions A, B, C and D (A-D corresponding to the order in which the samples came off the anionic exchange column, A first), plotted against mean pI value.

(a)

Epo Fraction	Mean pI value	Epo bioactivity (IU/mg)	95% Confidence Intervals (IU/mg)
Starting material	-	131,610	111,206-155,758
A	3.30	205,949	158,219-268,071
B	3.18	204,951	170,765-245,980
C	3.13	266,520	207,558-342,232
D	3.06	276,293	228,792-333,649

(b)



from the column and the order in mean pI values. Activities evaluated by the normocythaemic assay matched the expected order more closely, being in the order  $A=B<C<D$ . This is the trend one might have expected on the basis of time of fraction elution or mean pI alone, the reasoning being that fractions which come off the column later - due to a higher negative charge, indicating a greater level of sialylation and consequently a more acidic mean pI - are the most active. Although the two fractions eluted first (Fractions A and B) were extremely close in terms of activity, it was Fraction B that recorded a slightly lower figure and not the other way round. The results of the polycythaemic Epo assay did not follow the expected trend but it must be taken into account that both the normocythaemic and polycythaemic assay results, despite being the combined estimates of two potencies of independent experiments, could not be distinguished with statistical significance since the 95% confidence intervals were so wide. It is worth noting, that, as mentioned in Section 1.10, of the two *in vivo* assays, the normocythaemic assay, which gave results which more closely followed the expected trend, is considered the more robust in terms of precision and accuracy.

Recorded *in vivo* activity was higher for each of the four Epo fractions than it was for the starting material. The possibility remains that, while the concentration was in each case determined by ELISA (Section 2.3.12), the absolute concentration differed from that of the starting material.

#### 3.3.3.4. Glycoanalysis

Glycoprotein sugars can be analysed by a variety of methods, each of which provides a different level of information:

1. Monosaccharide analysis;

2. Glycan analysis - enzyme or chemical release of N-/O-glycans and analysis by various types of HPLC, followed by identification by mass spectrometry or enzyme microsequencing using exo-glycosidases;
3. Glycopeptide analysis – identification of the site of glycosylation by amino acid analysis.

Technique 2 was used to analyse the sugars liberated from Epo Fractions A-D.

Fig. 3.3.18. shows the graphs of the derivatised oligosaccharides from each Epo Fraction (A-D) as they eluted from a normal-phase (Amide-80) HPLC column. Mass spectrometry was used to identify which sugars were contained within each peak.

Relative amounts of oligosaccharide in each fraction were quantified by measuring the areas under the peaks and expressed as percentage of the total glycans (see

Table 3.3.5.). A second table (Table 3.3.6) was drawn up containing the moles of exposed galactose residues, sialic acids and extra N-acetylactosamine

(Gal $\beta$ 1 $\rightarrow$ 4GlcNAc $\beta$ 1 $\rightarrow$ 3) repeats per mole of glycan found within each fraction.

This was calculated using the following formula;

$$\sum \frac{(\% \text{ glycan structure})(\text{the number of residues conferred by glycan structure in question})}{100}$$

Proportions of tetra-sialylated tetra-antennary (including or excluding extra N-acetylactosamines), tri-antennary and bi-antennary sugars were also included in Table 3.3.6.

The fractions all contained high levels (about 63-67%) of tetra-antennary carbohydrates, around half of which (47-60%) were poly-lactosaminylated. Just under half (34-42%) of the tetra-antennary carbohydrates were fully sialylated and there was a tendency for tetra-antennary content (irrespective of tetrasialylation or

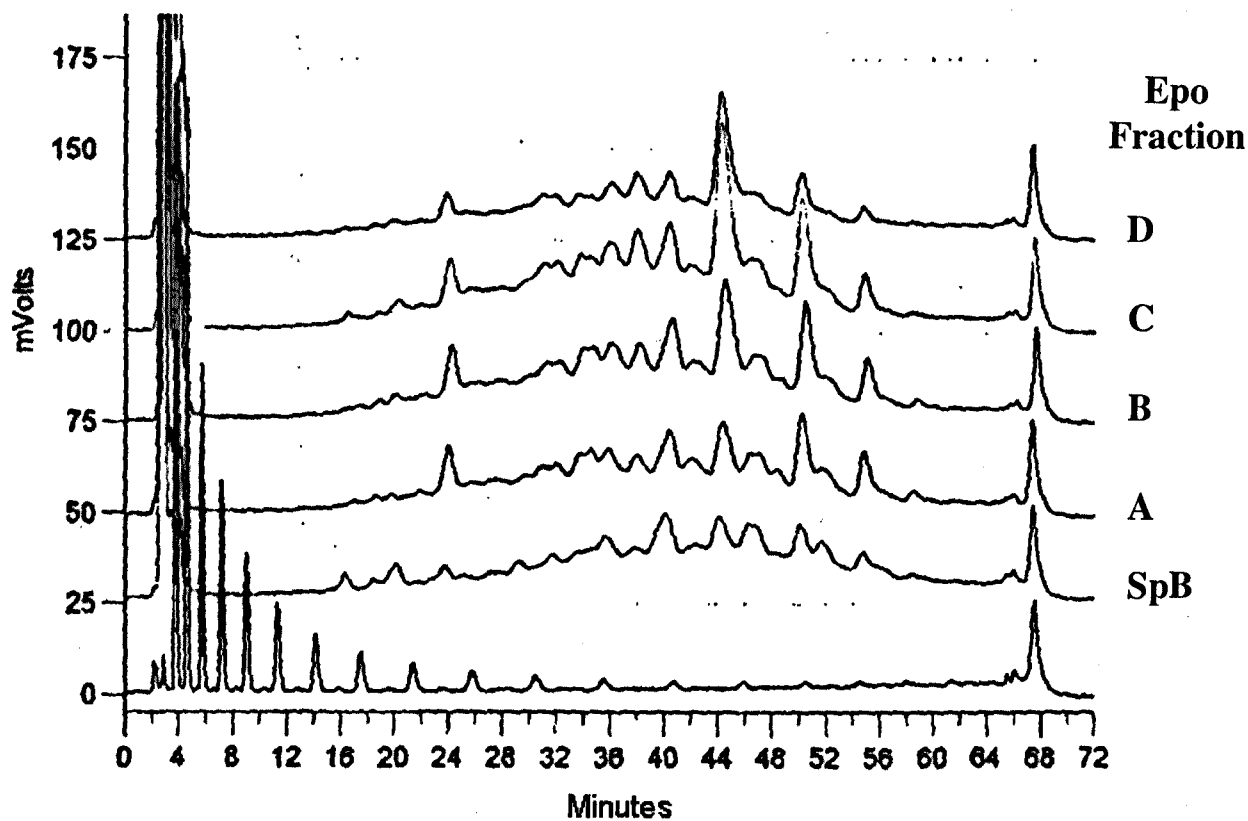


Fig. 3.3.18. Glycan profiles of the ion-exchange HPLC-separated Epo Fractions A to D carried out by Dr Yuen (NIBSC), as described in Section 2.3.14.

Please note: sample SpB referred to in Section 3.3.2. was included for comparison purposes.

Table 3.3.5. (Facing page) Table of percentages of each type of carbohydrate in Epo Fractions A to D, calculated by measuring the area below each peak in Fig.

### 3.3.18.

The number of sialic acids, exposed galactose residues and N-acetylactosamines conferred by each carbohydrate structure are also included.

N-glycan fraction	Proposed structure (number of exposed galactose residues) [number of N-acetylglucosamines]	Galactose Residues Exposed	Sialic Acids	Extra N-acetyl lactosamines	Fraction A	Fraction B	Fraction C	Fraction D
					% total	% total	% total	% total
1	?				0.8	0.9	1.4	1.1
2	Monosialylated bi-antennary,	1	1	0	1.8	1.4	2.0	2.8
3	?				0.9	2.1	1.4	1.6
4	Disialylated bi-antennary	0	2	0	2.3	5.2	4.5	4.7
5	?				6.7	7.4	7.4	6.6
6	?				3.3	3.4	2.8	3.3
7	Disialylated tri-antennary	1	2	0	7.3	3.1	3.5	4.5
8					3.9	4.1	3.8	4.0
9	Trisialylated tri-antennary	0	3	0	8.2	8.1	6.9	6.6
10	Disialylated tetra-antennary	2	2	0	6.7	6.9	7.4	7.1
11	Disialylated tetra-antennary + N-acetylglucosamine + 2 N-acetylglucosamine	0	2	1	5.8	6.5	8.1	8.3
12	Trisialylated tetra-antennary	1	3	0	9.3	9.6	8.8	8.3
13	Trisialylated tetra-antennary & unidentified tetrasialylated tetra-antennary structure				6.0	5.1	4.7	4.3
14	Tetrasialylated tetra-antennary	0	4	0	9.6	12.3	17.0	18.5
15	Trisialylated tetra-antennary + N-acetylglucosamine	1	3	1	7.2	6.4	5.4	4.9
16	Trisialylated tetra-antennary + N-acetylglucosamine	1	3	1	2.8	2.0	1.9	2.4
17	Tetrasialylated tetra-antennary + N-acetylglucosamine	0	4	1	7.6	7.7	8.0	6.1
18	Mix of tetrasialylated tetra-antennary + N-acetylglucosamine & trisialylated tetra-antennary + 2 N-acetylglucosamine				4.0	3.0	2.3	2.4
19	Tetrasialylated tetra-antennary + 2 N-acetylglucosamine	0	4	2	4.6	3.5	2.8	2.0
20	?				0.7	0.4	0.2	0.4



Table 3.3.6. (Facing page) Table of the proportions of each type of saccharide or oligosaccharide structure in Epo Fractions A to D.

Oligosaccharide structures (in percentages) were calculated by adding up the relevant N-glycan fractions. Saccharide proportions (in terms of moles per mole of glycan) were calculated by multiplying the percentage of each N-glycan fraction (Table 3.3.5.) by the number of exposed galactose residues, sialic acids or extra N-acetylglucosamine moieties conferred by the N-glycan fraction's structure (as laid out in Table 3.3.5.), taking the sum for all N-glycan fractions and dividing by 100 (see Section 3.3.3.4. for equation). Mean pI units are provided also (repeated from Table 3.3.4).

<b>Proposed structure</b>	<b>Epo Fraction A</b>	<b>Epo Fraction B</b>	<b>Epo Fraction C</b>	<b>Epo Fraction D</b>
Tetrasialylated tetra- antennary (including extra N- acetyllactosamines)	21.8 %	23.5 %	27.8 %	26.6 %
Tetrasialylated tetra- antennary (excluding extra N- acetyllactosamines)	9.6%	12.3 %	17.0 %	18.5 %
Tetra-antennary (including extra N- acetyllactosamines)	63.6 %	63 %	66.4 %	64.3 %
Tetra-antennary (excluding extra N- acetyllactosamines)	25.6 %	28.8 %	33.2 %	33.9 %
Tri-antennary	19.4 %	15.3 %	14.2 %	15.1 %
Bi-antennary	4.1 %	6.6 %	6.5 %	7.5 %
Exposed galactose residues	0.457	0.404	0.402	0.411
Sialic Acids	2.235	2.253	2.368	2.33
Extra N- acetyllactosamines	0.326	0.296	0.29	0.257
Mean pI value	3.30	3.18	3.13	3.06

N-acetyllactosaminylation) to increase in successive eluted fractions. The proportion of extra N-acetyllactosamines, by contrast, decreased.

The proportion of tri-antennary oligosaccharide chains was less than 20% and tended to decrease in successive eluted fractions; the figure for bi-antennary chains was even lower (less than 10%) but tended to increase.

Epo Fraction A had the highest number of galactose-terminated branches (around 0.45 moles per mole of glycan), whereas the other three fractions showed very little fraction-to-fraction variation (they were all in the range of 0.40-0.41 moles per mole of glycan). For pharmaceutical Epo, the amount of galactose exposure in the different eluted fractions did not follow a recognisable trend.

#### **3.3.3.5. Unmodified AS-E2 and AS-E2/ECL bioassays**

Proliferation curves obtained when testing Epo Fractions A-D in the AS-E2/ECL bioassays were extremely similar and so potencies (whether from the unmodified AS-E2 or the AS-E2/ECL bioassay) were derived from three independent experiments using the WRANL program and then combined using the COMPOT program (Section 2.2.4.1.). Epo Fraction A was selected as the standard with which to calculate relative potencies of the other samples. The combined estimates of potency are set out in Table 3.3.7a for the unmodified AS-E2 bioassay and 3.3.7b for the AS-E2/ECL bioassay. A representative graph from each of the two types of assay (AS-E2 and AS-E2/ECL) is shown in Fig. 3.3.19 (a and b respectively).

Table 3.3.7. Table of the combined estimate of potencies of Epo

Fractions A to D (derived from three independent experiments).

(a) by unmodified AS-E2 bioassay.

(b) by AS-E2/ECL bioassay.

(a)

<b>Epo Fraction</b>	<b>Potency of sample relative to Fraction A</b>	<b>95% Confidence interval</b>
A	1	-
B	1.023	0.995 – 1.052
C	1.026	0.998 – 1.055
D	1.014	0.995 – 1.035

(b)

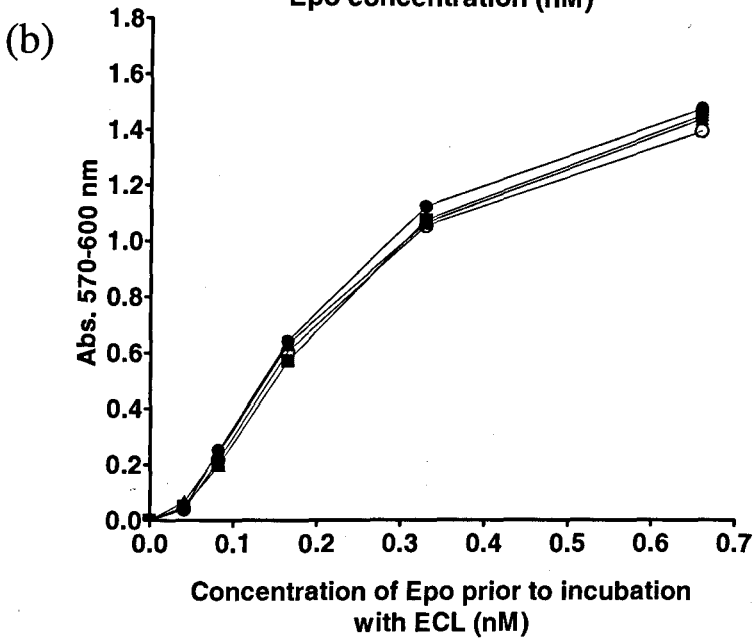
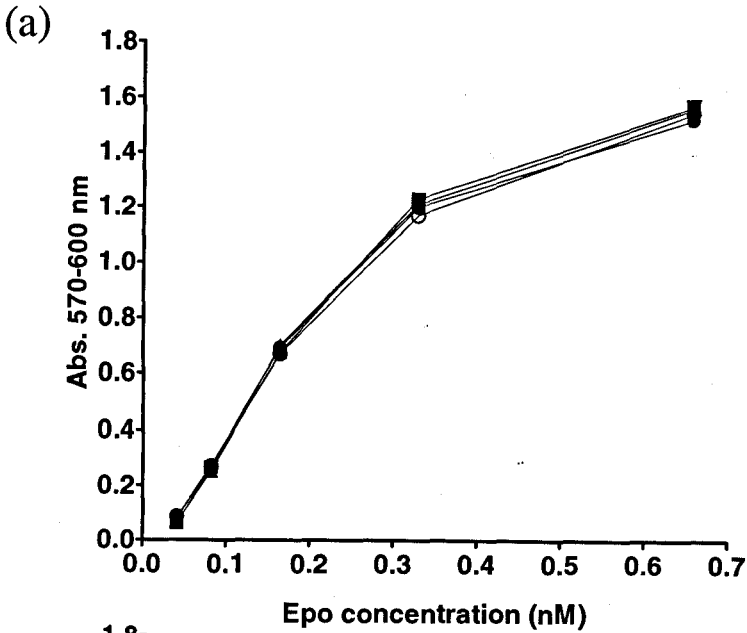
<b>Epo Fraction</b>	<b>Potency of sample relative to Fraction A</b>	<b>95% Confidence interval</b>
A	1	-
B	1.068	1.034 – 1.102
C	1.136	1.100 – 1.173
D	1.040	1.007 – 1.074

Fig. 3.3.19. (Facing page) Proliferative response of AS-E2 cells to Epo Fractions A to D (graphs representative of 3 independent experiments).

(a) by unmodified AS-E2 bioassay.

(b) by AS-E2/ECL bioassay

Fraction A (■), B (▲), C (●) and D (○). In (b), units along the x-axis represent the concentrations prior to treatment with ECL. Data represent mean  $\pm$  s.e.m. of triplicated samples (where an error bar is not shown, it lies within the dimensions of the symbol).



The four Epo Fractions, A-D, were statistically indistinguishable in the unmodified AS-E2 bioassay, with Fractions B, C and D all having 95% confidence intervals of between 0.99 and 1.05.

In the AS-E2/ECL bioassay, Epo Fractions B-D were more potent than Epo Fraction A, to a statistically significant degree ( $P < 0.05$ ), but not statistically different from each other. If using a decrease in mean pI as an indication of increase in sialylation, and by consequence potency, Epo Fractions A, B and C (mean pI values of 3.296, 3.18 and 3.126 respectively) followed the expected trend of increasing in relative potency (if A was set as 1, B was 1.068 and C was 1.136). Epo Fraction D did not fit in with this pattern, however, having a potency lower than both B and C (1.04), despite its mean pI value being the lowest, at 3.063. Consequently, it would perhaps be expected to have the highest number of sialic acids - and therefore (but not necessarily), the lowest number of exposed galactose residues - and be the least likely to be depleted in the agarose-conjugated ECL step. Surprisingly, glycoanalysis registered Fraction D as having more exposed galactose residues than Fractions B and C (see Table 3.3.6.) and so the observed lower activity was in agreement in that respect.

The AS-E2/ECL assay has been rationally designed to be responsive to the exposure of galactosyl residues. Sample set 1 proves that it is. Here, however, there is not a clear-cut relationship between mean pI and the number of exposed galactoses, and therefore there cannot be expected to be one between AS-E2/ECL activity and pI. Also, unlike sample set 1, there was no strongly defined relationship between pI and *in vivo* activity. So for this sample set derived from a pharmaceutical batch of Epo, it can be postulated that whatever was causing the isoform distribution, and presumably variation in activity, was not to do with exposed galactose residues.

### 3.3.3.6. Correlation of *in vivo* and *in vitro* bioactivity with non-biological data (IEF and glycan mapping)

Pearson correlation analysis was performed using Minitab to compare the results obtained by the two *in vivo* assays with the proportions of each sugar deduced in Table 3.3.6. The results are in Table 3.3.8. Before commenting on the correlations, it must be mentioned that, because of the small sample size, these statistics must be interpreted with caution. In addition, the results of the polycythaemic and the normocythaemic Epo mouse bioassays correlated with a Pearson correlation coefficient of 0.764. This is perhaps not as high as would be expected from two assays measuring the same property of the same hormone. The lower than expected correlation may be attributed to the small number of sample sets being measured, the relative similarity between the samples being tested in terms of mean pI, and the low level of precision afforded by the method used to test them.

There was also a problem in that N-glycan fractions 13 (trisialylated tetra-antennary and unidentified tetrasialylated tetra-antennary structures) and 18 (mix of tetrasialylated tetra-antennary structures including one N-acetyllactosamine, and trisialylated tetra-antennary structures including two N-acetyllactosamines) identified by glycoanalysis (see Table 3.3.5.) were made up of more than one species of oligosaccharide, thereby adding to the uncertainty as to what the proportions of each individual sugar are. These two N-glycan fractions were omitted from calculations assessing saccharide content but could nevertheless make crucial differences to the figures in Table 3.3.6.



	Polycythaemic mouse assay	Normocythaemic mouse assay	Mean pI value
	R value (P-value)		
Tetrasialylated tetra-antennary carbohydrates (extra N- acetyllactosamines included)	0.777 (0.223)	0.925 (0.075)	-0.872 (0.128)
Tetrasialylated tetra-antennary carbohydrates (extra N- acetyllactosamines excluded)	0.617 (0.383)	0.959 (0.041)	-0.964 (0.036)
Tetra-antennary carbohydrates (extra N-acetyllactosamines included)	0.982 (0.018)	0.938 (0.062)	-0.439 (0.561)
Tetra-antennary carbohydrates (extra N-acetyllactosamines excluded)	0.620 (0.380)	0.736 (0.264)	-0.967 (0.033)
Tri-antennary carbohydrates	-0.387 (0.613)	-0.645 (0.355)	0.870 (0.130)
Bi-antennary carbohydrates	0.169 (0.831)	0.673 (0.327)	-0.959 (0.041)
Exposed galactose residues	-0.229 (0.771)	-0.507 (0.493)	0.811 (0.189)
Sialic acids	0.863 (0.137)	0.930 (0.070)	-0.798 (0.202)
Extra N-acetyllactosamines	-0.254 (0.746)	-0.806 (0.194)	0.973 (0.027)
Mean pI value	-0.4 (0.6)	-0.855 (0.146)	-

Table 3.3.8. Measurement of association between the *in vivo* assays

(polycythaemic and normocythaemic) and carbohydrate proportions identified by glycoanalysis, using Pearson correlations.

Subject to these caveats, it may perhaps be said that the normocythaemic assay can provide the most useful results since, as mentioned in Section 3.3.3.3., it is the more accurate and precise of the two *in vivo* assays.

The proportion of tetrasialylated tetra-antennary sugars has been shown to correlate strongly with the *in vivo* biological activity of Epo (Yuen *et al*, 2003). What is immediately apparent is that the *in vivo* assays described here, in particular the normocythaemic assay, exhibit a positive correlation with the proportion of tetra-antennary carbohydrates, regardless of tetrasialylation or poly-lactosaminylation, the majority of the correlation values being above 0.7. The fact that this correlation held true even if sialylation was not taken into account may be explained by the fact that if there is a tendency to have more branching then there is also a tendency to have more sialylation, as indicated by the positive correlation between tetrasialylated tetra-antennary and all tetra-antennary sugar content ( $R=0.821$ ,  $P=0.179$ ).

There was no correlation between *in vivo* activities and tri-antennary carbohydrate content: R values for both assays were  $>-0.7$ , in agreement with Yuen *et al* (2003) who found no positive correlation between these two variables. It was the same case for comparisons with bi-antennary content, i.e. R values were  $<0.7$ .

Surprisingly, there was little to no correlation between exposed galactose residues and either of the *in vivo* Epo bioassays ( $R=-0.507$  and  $R=-0.229$  for the normocythaemic and polycythaemic assays respectively). Mean pI value negatively correlated ( $R=-0.855$ ,  $P=0.146$ ) and sialic acid positively correlated ( $R=0.93$ ,  $P=0.070$ ) with the normocythaemic assay, pI and sialic acid being two variables that are arguably, although not necessarily, linked to galactose exposure (it being possible for there to be low levels of both branching and sialylation and yet still sialylation of all termini).

The presence of extra N-acetylglucosamines appeared to have an adverse effect on biological activity as measured by the normocythaemic assay ( $R=-0.855$ ,  $P=0.146$ ). The *in vivo* results in Figs. 3.3.16-17. and the figures in Table 3.3.6. then underwent correlation analysis with the results obtained by the two *in vitro* cell-based assays (see Table 3.3.9.). The AS-E2/ECL bioassay did not correlate with either the normocythaemic ( $R=0.467$ ,  $P=0.533$ ) or the polycythaemic ( $R=0.615$ ,  $P=0.385$ ) mouse Epo bioassay, although it did negatively correlate with the number of exposed galactose residues ( $R=-0.790$ ,  $P=0.210$ ), the variable the assay was designed to assess. The unmodified AS-E2 assay correlated even more strongly with the number of exposed galactose residues than did the AS-E2/ECL assay ( $R=-0.964$ ,  $P<0.05$ ) but this is not reliable as the relative potencies were statistically indistinguishable from each other.

### **3.4. Discussion**

One of the aims of this project was to set up a cell-based bioassay for Epo, one step of which involved the incorporation of a plant lectin whose role was to mimic the action of the liver. Plant lectins are proteins with highly specific sugar-binding properties. One such lectin, *Erythrina crista-galli* lectin (ECL), has the same binding specificity (i.e. galactose residues) as the ASGP-R (Bhattacharyya *et al*, 1989), although the two proteins do not share any sequence homology (Loris, 2002). In this assay, any Epo displaying carbohydrate structures terminating in galactose residues was bound to ECL-agarose and then removed by centrifugation (as outlined in Fig. 3.1.1.). The resulting supernatant was then tested in a cell proliferation assay using the AS-E2 Epo-dependent cell line. The rationale was that samples containing a higher proportion of desialylated Epo would be depleted of more Epo molecules and thus

Table 3.3.9. Measurement of association between the *in vitro* assays (AS-E2 and AS-E2/ECL), *in vivo* assays (polycythaemic and normocythaemic) and carbohydrate proportions identified by glycoanalysis, using Pearson correlations.

	AS-E2 bioassay	AS-E2/ECL bioassay
	R value (P-value)	
Polycythaemic mouse assay	0.268 (0.732)	0.615 (0.385)
Normocythaemic mouse assay	0.377 (0.623)	0.467 (0.533)
Exposed galactose residues	-0.964 (0.036)	-0.790 (0.210)
Sialic acids	0.604 (0.396)	0.751 (0.249)
Mean pI value	-0.650 (0.350)	-0.511 (0.489)

stimulate less cell proliferation. All samples were also tested in the unmodified AS-E2 bioassay to verify that initial concentrations and activities were the same, and that differences in the AS-E2/ECL bioassay were not due to such discrepancies.

The first sample set subjected to the AS-E2/ECL assay was Epo desialylated using agarose-conjugated neuraminidase. Using a solid-phase version of this enzyme meant that termination of the reaction could be controlled more precisely (only a simple spin-down step is required) and there was no need to use inhibitors.

The standard cell-based assay employed alamarBlue to measure metabolic activity. It was not strictly a proliferation assay in the same way as the tritiated thymidine incorporation or BrdU proliferation methods, which both measure DNA synthesis.

However, the alamarBlue assay was seen as a proliferation assay in the sense that an increase in metabolic activity was indicative of a greater number of cells, which could be due to an increase in proliferation or an increase in cell survival (Epo stimulating both proliferation and survival pathways (reviewed in Ghaffari *et al*, 2003)).

The AS-E2/ECL bioassay included a pre-incubation step with solid-phase lectin, as simply adding ECL to the wells during the course of the assay did not give the same results. ECL did not act as a competitive antagonist (Fig. 3.3.8.). This was consistent with findings that residues involved in receptor binding do not overlap glycosylation sites. Asn<sup>147</sup>, Arg<sup>150</sup>, Gly<sup>151</sup>, Thr<sup>40</sup>-Lys<sup>52</sup> form the first, high-affinity receptor binding site and Val<sup>11</sup>, Arg<sup>14</sup>, Tyr<sup>15</sup>, Ser<sup>100</sup>, Arg<sup>103</sup>, Ser<sup>104</sup> and Leu<sup>108</sup> form the second, low-affinity receptor binding site (Cheetham *et al*, 1998). Site-directed mutagenesis studies found the following four regions to be important for bioactivity: amino acids 11-15, 45-51, 100-108 and 147-151 (Elliott *et al*, 1997), none of which were glycosylation sites.

The *in vivo* mouse assay for Epo was extremely sensitive to desialylation, as a comparison between untreated Epo and the slightly desialylated Epo sample (t=20 min) will readily show. Loss of sialic acids corresponding to a shift of just 0.3 mean pI units resulted in a 73% reduction in biological activity using the polycythaemic mouse bioassay (see Fig. 3.3.7.). The modified *in vitro* AS-E2/ECL bioassay was less sensitive, measuring only a 29% decline in biological activity. We can speculate as to the reasons for this discrepancy. The liver is one of the largest organs in the body and constitutes a major reservoir of asialoglycoprotein receptors. Exposure of just 1 or 2 galactose residues per molecule of Epo could be enough to allow the liver to remove Epo from the circulation. Indeed, Van Den Hamer *et al* (1970) predicted that ferroxidase (a copper transport glycoprotein found in the blood) would require only two galactose residues to be exposed in order to effect clearance. In the *in vitro* agarose-conjugated ECL system, however, a critical number of galactose residues appeared to need to be exposed on Epo before it was efficiently removed but once that number was reached, the system became especially adept at depleting the solution of such molecules.

A similar difference in loss in sialylation between the Epo Fractions separated by anion-exchange chromatography (indicated by a 0.24 mean pI unit difference between Fractions A and D) did not result in such a large drop in *in vivo* activity. Instead of the 73% drop observed for Epo sample t=20 min compared to t=0 min, there was only a 25.5% drop (by normocythaemic assay) between Fractions A and D. It may be that changes in sialylation of Epo molecules at a certain threshold level of sialylation, around pI values of 3.0 units, do not affect activity to such an extent. Morimoto *et al* (1996) found that there was no further increase in the activity of Epo fractions

separated by anion-exchange which had more than 12.4 moles sialic acid per mole of Epo.

In the anion-exchange separated third sample set, an increased number of extra N-acetylglucosamines coincided with decreased biological activity.

Poly N-acetylglucosamine repeats have, in addition to the number of exposed galactoses, been implicated in clearance by the ASGP-R (Fukuda *et al*, 1989). Doubts can be cast on this finding, though, as tomato lectin, having a preference for N-acetylglucosamine repeats, was used to isolate glycoproteins containing a higher proportion of them. This enriched fraction was then used in *in vivo* assays and found to be cleared more rapidly from the circulation, even though tomato lectin also tends to bind non-sialylated sugars rather than sialylated sugars (Merkle & Cummings, 1987). In the case of the anion-exchange separated Epo Fractions, N-acetylglucosamine repeats associated with exposed galactose residues with a correlation coefficient of  $R=0.702$  ( $P=0.298$ ). It could have been a matter of exposed galactose residues mediating clearance by the ASGP-R of the liver rather than anything to do with N-acetylglucosamine repeats.

Epo Fraction D had slightly more galactose residues exposed than Fraction C (approximately an extra 0.01 mole of galactose per mole of glycan) and fewer sialic acids, something which might not have been expected in the case of the most acidic sample (based on the IEF gel). The possibility cannot be ruled out, therefore, that unintentional sugar degradation, for instance by contamination with glycosidases, may have occurred in this fraction.

Each set of samples used in this study represented different types of material that might be encountered when assessing the quality of Epo for batch release. Sample set 1 represented Epo that might possibly have been exposed to neuraminidase (e.g. in

Table 3.4.1. Methods that can detect changes in isoform distribution and an assessment of how sensitive they were when performing tests on each of the three Epo sample sets.

	Sample set 1 (neuraminidase treated)	Sample set 2 (different culture and purification conditions)	Sample set 3 (ion-exchange fractionated pharmaceutical grade Epo)
<i>In vivo</i> assay	✓✓✓	✓✓	x
<i>In vitro</i> AS-E2 assay	x	x	x
<i>In vitro</i> AS-E2/ECL assay	✓✓	✓	x
IEF	✓✓	✓✓	✓✓
Glycan mapping	Not applied	✓✓✓	✓✓✓



the form of a culture medium/serum contaminant); sample set 2 represented Epo that had been generated by different culture and purification methods; and sample set 3 was intended to reflect the range of isoforms that could possibly be present in a single batch of pharmaceutical-grade Epo. Table 3.4.1. lists the methods available for detecting changes in isoform distribution and describes how, in terms of sensitivity, they performed during tests on each of the three sample sets. *In vivo* assays differentiated with statistical significance between the different samples within set 1. These types of assay were less successful in discriminating between the fractions in sample sets 2 and 3. This can be explained by these sample sets spanning a narrower range of mean pI values.

As expected, the unmodified AS-E2 assay failed to differentiate between the samples in any of the sample sets.

The modified AS-E2/ECL assay was able to reveal differences in activity between the samples within sample set 1 and, to a lesser extent, within sample set 2. The samples in sample set 3 were all but indistinguishable using this technique.

While IEF had the ability to detect variation within all sample sets, it was unable to provide the same level of detailed information as glycan mapping.

### **3.5. Conclusion**

A variety of methods are available for measuring or predicting Epo bioactivity. This chapter describes how a non-radioactive cell-based assay for Epo has been developed that incorporates an immobilised form of the galactose-binding plant lectin, ECL, thus rendering the assay sensitive to sialic acid content. This assay was tested alongside an established array of techniques to evaluate their sensitivity in distinguishing between a range of different Epo sample sets: experimentally desialylated Epo; Epo produced by

different culture and purification procedures; and a batch of pharmaceutical-grade Epo separated into fractions by anion-exchange HPLC. The strategy of developing an Epo-dependent sialylation-sensitive cell-based bioassay worked particularly well when assaying sample set 1, which was designed to vary only in terms of sialylation level. Sample set 3, however, revealed a limitation in that although pharmaceutical Epo separated into fractions by anion-exchange HPLC could be differentiated in terms of isoform distribution by IEF, it was not distinguishable by AS-E2/ECL assay. The *in vivo* assays fared little better in this respect, and sialic acid/galactose content did not correlate well with mean pI. The invariance in galactose content provided an explanation for the insensitivity of the modified Epo bioassay.

## **Chapter 4. Development of a sialylation-sensitive cell-based bioassay; stable transfection of an Epo-dependent cell line with the asialoglycoprotein receptor**

### **Summary and Overview**

Chapter 4 describes the development of a sialylation-sensitive *in vitro* assay by stable transfection of an Epo-dependent cell line with a vector encoding the asialoglycoprotein receptor. It features the following sequence of studies:

- (i) Isolation of cDNAs encoding the two subunits of the asialoglycoprotein receptor (ASGP-R) and vector construction (Sections 4.2.1., 4.2.2., 4.3.1., and 4.3.2.);
- (ii) Detection of vector-driven ASGP-R expression by Western blot and fluorescence microscopy (Sections 4.3.4. and 4.3.5.);
- (iii) Comparison of the potencies of intact and desialylated Epo obtained in assays employing untransfected/empty vector-transfected and asialoglycoprotein receptor-transfected Epo-dependent cells (Section 4.3.6.1);
- (iv) Demonstration of the galactose-inhibability of the sialylation-sensitive effect (Section 4.3.6.2.);
- (v) Discussion of the results (Section 4.4).

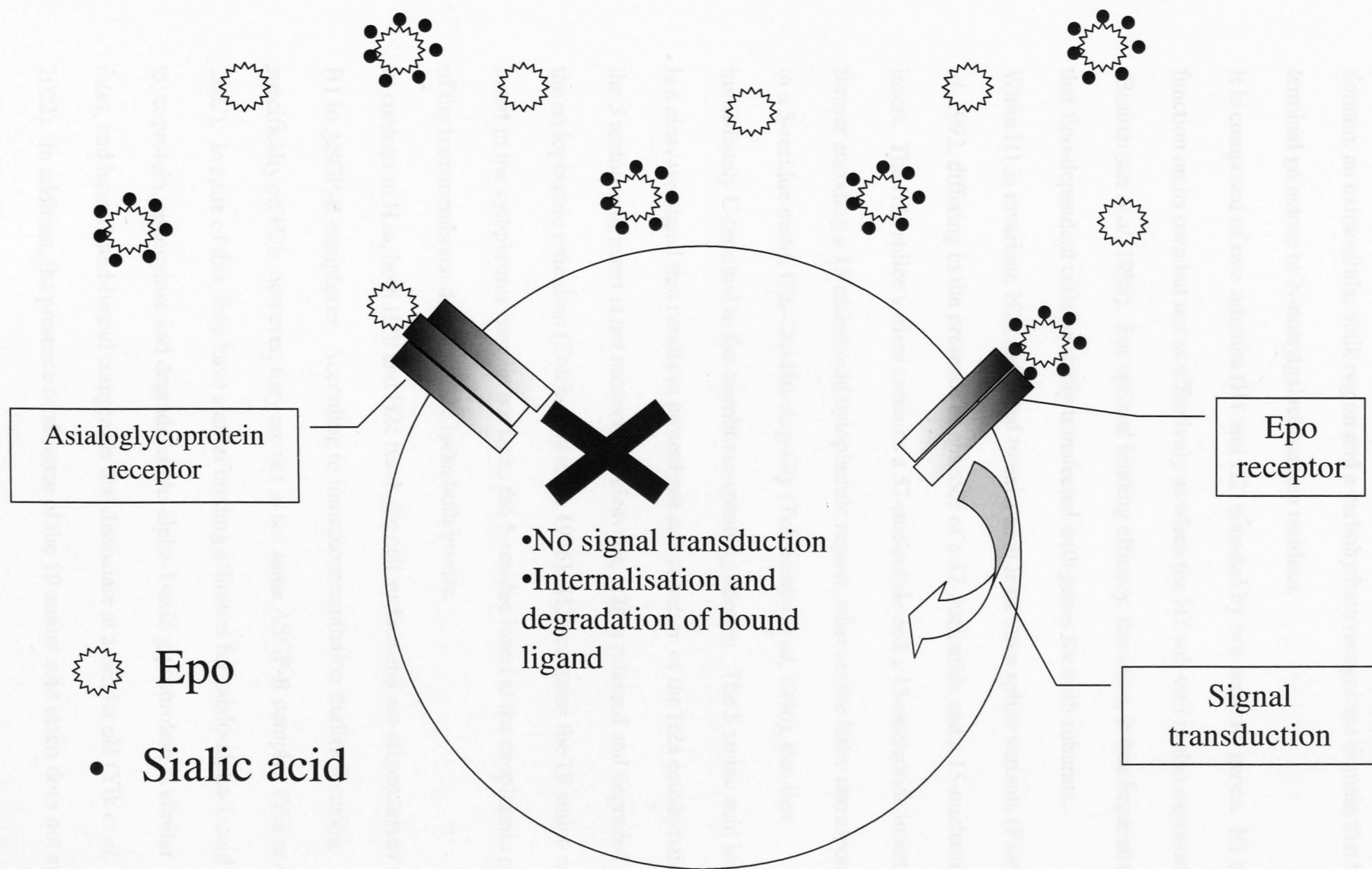
### **4.1. Introduction and aims of the study**

While the AS-E2/ECL lectin bioassay described in Chapter 3 provides evidence to back up the principle that rational manipulation of *in vitro* bioassays can in some circumstances allow prediction of *in vivo* activity, it does have a number of

drawbacks: the lectin pre-incubation step needed prior to serial dilutions of samples being made brings with it additional inherent errors; lectin-to-agarose coupling efficiency may well vary between batches; and plant lectin is arguably "artificial", bearing little relevance to *in vivo* physiological processes. The aim of the work described in this chapter was to copy more closely what happens in the body by introducing the liver receptor responsible for clearing incompletely sialylated glycoproteins from the circulation into an Epo-dependent cell line. Such a receptor would effectively sequester and internalise Epo containing exposed galactose residues, thus competing with the Epo-R and precluding this population of cytokine molecules from transmitting a proliferation signal (see Fig. 4.1.1. for a schematic representation of the decoy strategy). Decoy receptors already exist in nature; they include a truncated Epo-R lacking cytosolic signal transduction domains (Nakamura *et al*, 1992; Nakamura & Nakauchi, 1994), the murine Interleukin-1 type II receptor, which has been hypothesised to be a surface receptor that blocks the ability of cells to respond to IL-1 (Donaldson *et al*, 1998), and TRID (TRAIL receptor without an intracellular domain), a receptor for TRAIL (Tumor necrosis factor-related apoptosis-inducing ligand) that consists of a TRAIL-binding extracellular domain and a transmembrane domain but that lacks an intracellular domain. The latter is thought to act as a decoy receptor for TRAIL, thus protecting the cell from TRAIL-induced apoptosis (Pan *et al*, 1997).

As stated in the introduction, the ASGP-R is a type II membrane receptor. Instead of having an N-terminal signal sequence that navigates the polypeptide through the cell-surface membrane, as in type I receptors, the ASGP-R contains a transmembrane domain that acts as an internal signal sequence, directing the C-terminal domain to appear on the outside of the cell (Spiess & Lodish, 1986). Consequently, its structure

Fig. 4.1.1. (Facing page) Schematic representation of the decoy receptor strategy. Epo, regardless of the sialic acid content, binds the Epo-R, but Epo lacking terminal sialic acids will also bind the asialoglycoprotein receptor (ASGP-R), effecting the removal of this population of molecules from the surrounding medium and lowering the concentration.



consists of a cytosol-facing NH<sub>2</sub>-terminal domain, a single-span transmembrane domain, an extracellular stalk region and a carbohydrate recognition domain that binds terminal galactose or N-acetylgalactosamine residues.

It is composed of two subunits (H1 and H2) encoded by two separate genes. H1 can function on its own but not as effectively as when the H2 sub-unit is also expressed (Braiterman *et al*, 1989). For optimal binding efficacy, therefore, it was imperative that Epo-dependent cells be stably transfected with genes for both subunits.

While H1 is invariant, H2 is reported to exist as at least three splice variants (Paietta *et al*, 1992, differing in the presence or absence of a 57-nucleotide and a 15-nucleotide insert. The H2a splice variant contains a 57-nucleotide and a 15-nucleotide insert, the former encoding a 19 amino acid cytoplasmic region, whereas the latter corresponds to a 5-residue motif, Glu-Gly-His-Arg-Gly (Tolchinsky *et al*, 1996), that lies immediately C-terminal to the membrane-spanning domain. The 5 amino acid insert is a cleavage signal that results in proteolysis and secretion of the H2a ectodomain. If the 5 amino acid insert is not removed by cleavage, H2a is retained and degraded in the endoplasmic reticulum (Tolchinsky *et al*, 1996). H2b contains the 19 amino acid insert in the cytoplasmic domain but lacks the 5-residue insert at the exoplasmic end of the transmembrane domain. H2c lacks both inserts.

In contrast to H2a, both H2b and H2c reach the cell surface and are oligomerised with H1 in ASGP-R complexes. According to immunoprecipitation studies focusing specifically on H2b, however, they are not in the same ASGP-R complex (Yik *et al*, 2002). In spite of this, they have similar binding affinities for asialo- $\alpha$ -1-acid glycoprotein, endocytose and degrade asialo- $\alpha$ -1-acid glycoprotein at similar rates, and have ligand-bound complexes that dissociate at a similar pH (Yik *et al*, 2002). In addition, the presence or absence of the 19 amino acid insert does not affect

palmitoylation of H2 at cysteine residues 54 and 58. Yik *et al* speculate that H2b and H2c may be involved in different endocytic pathways, of which there are two: the State 1 and the State 2 pathways. In the State 2 pathway, internalised ligand is dissociated more rapidly (Oka & Weigel, 1983). It is thought that phosphorylation increases the rate of endocytosis and that the 57-nucleotide insert, found in H2b but not in H2c, is necessary for serine<sup>12</sup> phosphorylation (Paietta *et al*, 1992a; Geffen *et al*, 1991). Accordingly, since the H2b subunit is thought to mediate a faster rate of endocytosis, it was chosen, along with the H1 subunit, for co-expression in an Epo-dependent cell line.

## **4.2. Experimental procedures**

### **4.2.1. PCR-amplification of first-strand cDNA and cloning of the ASGP-R**

Messenger RNA was extracted from the liver cell line Hep-G2 (Knowles *et al*, 1980), followed by first-strand cDNA synthesis as described in Sections 2.1.1. and 2.1.2. The ASGP-R H1 and H2 subunits were PCR-amplified with primers designed according to the published sequences in the GenEMBL database. The H1, H2a and H2c subunit Accession numbers are E12702, X55284 and E12703 respectively. Since the H2b subunit had not been submitted to the database, it was deduced from the literature (Yik *et al*, 2002). The same primer pair is suitable for all the H2 splice variants, as any differences in sequence are some distance away from the 5' and 3' ends.



*The following primers were used:*

Primer name	Primer sequence	Restriction site
H1-5' (forward)	5'-gacgatgctagccagccctatcatgaccaaggagta-3'	- <i>Nhe</i> I underlined
H1-3' (reverse)	5'-gacgatacgcggtttaaaggagaggtggctcctggct-3'	- <i>Mlu</i> I underlined
H2-5' (forward)	5'-gacgattctagagggcccatcatggccaaggactt-3'	- <i>Xba</i> I underlined
H2-3' (reverse)	5'-gacgatgcggccgctcaggccacctcgccggtggca-3'	- <i>Not</i> I underlined

The primers had recognition sites for restriction enzymes introduced (underlined) that enabled ligation into the vector pIRES.

PCR-amplification of H1 produced an expected product of 875 bp. Amplification of H2 produced a product of approximately 933 bp (see Fig. 4.2.1. for the UV photographic record). These PCR products were ligated into pGEM-T (Promega) as described in Section 2.1.4., transformed into JM109 *E. coli* cells (Section 2.1.5.) and digested by the relevant restriction endonuclease (see Table above) and separated on 1% agarose gels (Section 2.1.9.) to check for correct-sized inserts.

#### **4.2.2. Identification of the H2b splice variant**

*Analytical PCR was performed on the H2 clones in order to identify the H2b splice variant;*

Name	Primer sequence
57-5' (forward)	5'-gggccccatcatggccaaggactt-3'
57-3' (reverse)	5'-gaggagctccatctggcaggccac-3'

Primers 57-5' and 57-3' enabled differentiation between clones in which the 57 bp insert was present (H2b) and those in which it was absent (H2a and H2c).

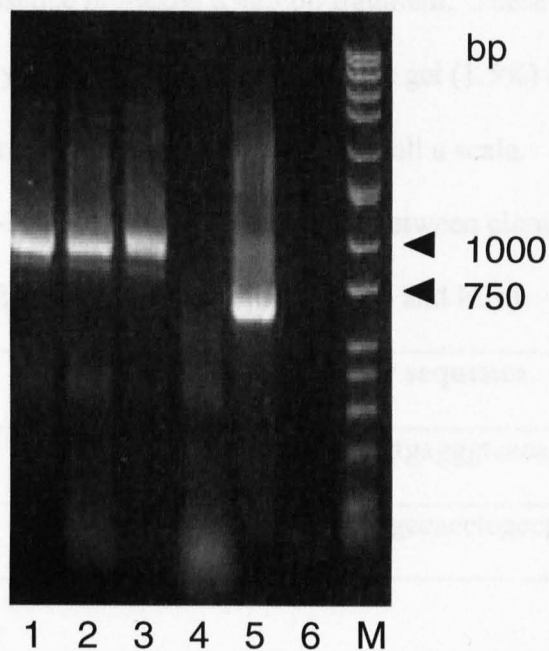


Fig. 4.2.1. PCR amplification of ASGP-R H1 (expected product size= $\sim$ 875 bp) and H2 (expected product size= $\sim$ 931 bp) subunits using the listed primers and HepG2 first-strand cDNA as a template. Ethidium Bromide-stained 1% agarose gel loaded as follows:

-Lane 1; primers H1-5' and H1-3' (2  $\mu$ M final concentration).

-Lane 2; primers H1-5' and H1-3' (20  $\mu$ M).

-Lane 3; primers H2-5' and H2-3' (2 $\mu$ M).

-Lane 4; primers H2-5' and H2-3' (20  $\mu$ M).

-Lane 5; positive control  $\beta$ -actin primers 2  $\mu$ M.

-Lane 6; beta actin primers alone.

Primer 57-5' recognised a region 64 bp upstream of the potential site of the 57 bp insert, while primer 57-3' formed complementary base pairs with a site 340 bp downstream.

The presence of the 57 bp insert in a clone resulted in a 480 bp PCR fragment being produced while its absence produced a 423 bp fragment. These PCR products were separated on a slightly higher percentage of agarose gel (1.5%) in order to facilitate differentiation between differences in size on so small a scale.

Primers 15-5' and 15-3' were used to differentiate between clones that contained the extra 15 bp insert (H2a) and those that did not (H2b and H2c).

Name	Primer sequence
15-5'	5'-aaagtgagggtcacagaggtgcac-3'
15-3'	5'-tcaggccacctcgccggtggcatt-3'

The strategy used for detecting the presence of the 57 bp insert could not be used for the 15 bp insert, since a difference of just 15 nucleotides is too small to be seen on an agarose gel. Instead, primer 15-5' was designed to recognise the extra 15 bp insert while primer 15-3' was complementary for a region 656 bases downstream of the mini-exon. If a clone contained the 15 bp insert, a 704 bp PCR fragment (including primers) was produced. Absence of a PCR fragment indicated absence of the 15 bp insert. There was admittedly a greater risk of false negative results (for instance if the PCR reaction failed) but, with this in mind, all clones were further verified by sequencing.

### **4.2.3. Plasmid construction**

Clones identified as containing the correct H1 and H2b sequences were sub-cloned into the pIRESblast mammalian expression vector (described in Section 2.2.3.2.).

This was achieved in two steps, the first being ligation into the pIRES vector (Clontech), resulting in the pIRES(H1-IRES-H2b) construct. The pIRES vector (as distinct from the pIRESblast vector) contains two multiple cloning sites separated by an Internal Ribosome Entry Site (IRES) sequence, its purpose being the co-expression of two genes of interest. This vector was not considered suitable for stable transfections, though, as the selectable marker is not coexpressed with the genes of interest.

*The second step was PCR-amplification of the entire tripartite cassette, designated H1-IRES-H2b, with primers incorporating ClaI and EcoRI restriction ends:*

Name	Primer sequence	Restriction site
<i>ClaI</i> -H1-5'	5'-gacgatatcgatcagccctatcatgaccaaggagta-3'	- <i>ClaI</i> underlined
<i>EcoRI</i> -H2b-3'	5'-gacgatgaattctcagccacctcgccggtggca-3'	- <i>EcoRI</i> underlined

Apart from having different restriction sites, primers *ClaI*-H1-5' and *EcoRI*-H2b-3' are identical to primers H1-5' and H2-3' respectively.

The ~2.4 Kbp *ClaI*-H1-IRES-H2b-*EcoRI* fragment produced by PCR (see Fig. 4.2.2.) was TA-cloned into PGEM-T, checked by nucleotide sequence analysis and then ligated into the multiple cloning site of pIRESblast between restriction sites *ClaI* and *EcoRI*. A clone with the correct insert was isolated and designated p[H1-IRES-H2b]IRESblast. An endotoxin-free maxi-prep batch of p[H1-IRES-H2b]IRESblast was prepared using the EndoFree Plasmid Maxi Kit (Qiagen) and used in stable transfections.

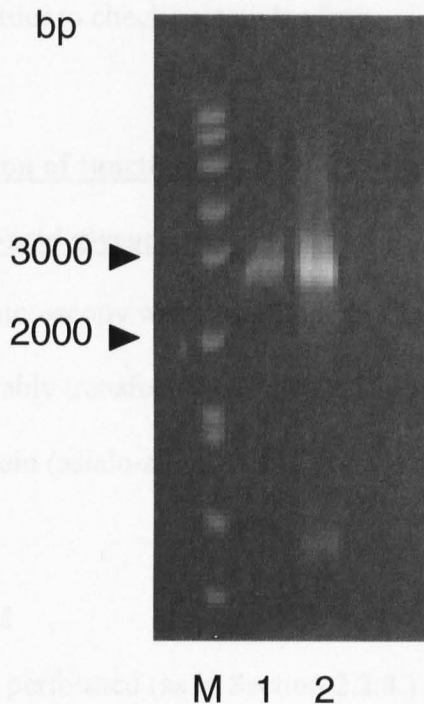


Fig. 4.2.2. PCR amplification of [ASGP-R-H1]-[IRES]-[ASGP-R-H2b] with *EcoRI* and *ClaI* restriction ends using the listed primers and pIRES(H1-IRES-H2b) as a template. Ethidium Bromide-stained 1% agarose gel loaded as follows:

-Lane 1; *ClaI*-H1-5' and *EcoRI*-H2b-3' primers (2  $\mu$ M final concentration).

-Lane 2; *ClaI*-H1-5' and *EcoRI*-H2b-3' primers (20  $\mu$ M).

Expected product size = ~2600 bp

#### **4.2.4. Expression of immunoactive ASGP-Rs: Western blotting**

Western blots using an anti-ASGP-R-H1 antibody (Calbiochem) were performed in order to check expression of the ASGP-R in stably transfected cells (Section 2.3.5.). A Western blot using an anti-tubulin antibody (Santa Cruz Biotechnology, USA) was carried out in order to check protein loading.

#### **4.2.5. Expression of functional ASGP-R: binding of fluorophore-labelled asialo-alpha-1-acid glycoprotein**

Fluorescence microscopy was carried out as described in Section 2.3.13. to determine whether cells stably transfected with the ASGP-R were capable of binding an asialoglycoprotein (asialo-alpha-1-acid glycoprotein).

#### **4.2.6. Bioassays**

Bioassays were performed (as in Section 2.2.4.) using the AS-E2 cells that had been stably transfected with the ASGP-R-H1 and -H2b subunits. Untransfected AS-E2 cells or AS-E2 cells stably transfected with empty vector were used as the control. In a set of experiments to investigate the effect of ASGP-R inhibitors on the cell assays, desialylated alpha-1-acid glycoprotein (120  $\mu$ M) or lactose (100 mM) were included in the assay medium at an excess concentration (100,000x in the case of desialylated alpha-1-acid glycoprotein).

#### **4.2.7. Plate plan**

Fig. 4.2.3. shows a typical plate plan used in the 96-well bioassays.

	1	0.5	0.25	0.125	0.0625	0.0625	0.125	0.25	0.5	1	
	U	U	U	U	U	U	U	U	U	U	
	1	0.5	0.25	0.125	0.0625	0.0625	0.125	0.25	0.5	1	
	U	U	U	U	U	U	U	U	U	U	
	1	0.5	0.25	0.125	0	0	0.125	0.25	0.5	1	
	U	U	U	U	U	U	U	U	U	U	
	1	0.5	0.25	0.125	0	0	0.125	0.25	0.5	1	
	T	T	T	T	T	T	T	T	T	T	
	1	0.5	0.25	0.125	0.0625	0.0625	0.125	0.25	0.5	1	
	T	T	T	T	T	T	T	T	T	T	
	1	0.5	0.25	0.125	0.0625	0.0625	0.125	0.25	0.5	1	
	T	T	T	T	T	T	T	T	T	T	

Fig. 4.2.3. Plate plan for stably transfected cell-based Epo bioassay. Wells marked “U” contain untransfected AS-E2 cells or empty vector-transfected cells, while wells marked “T” contain lectin-transfected AS-E2 cells. Blue wells contain dilutions of non-neuraminidase-treated Epo untreated with neuraminidase (sample t=0 min), whereas red wells contain neuraminidase-treated Epo (sample t=20 min, sample t=480 min or sample t=1800 min). The top dose (1) corresponds to 0.33 nM. Grey outer wells contain medium only.

## **4.3. Results**

### **4.3.1. Verification of p[H1-IRES-H2b]IRESblast by sequencing**

A map of the intended p[H1-IRES-H2b]IRESblast vector is shown in Fig. 4.3.1 along with the primers used to confirm by sequencing that the construct was correct.

### **4.3.2. The H2b splice variant was identified using analytical primers**

An issue encountered was the requirement to identify the H2b splice variant amongst the other possible variants. This was done using primer pairs 57-5'/57-3' and 15-5'/15-3' (Section 4.2.2.). Fig. 4.3.2. shows the splice variants of the ASGP-R-H2 sequence and the sites of primers used for splice variant identification. Analytical PCR was performed on ASGP-R-H2 cDNA-containing pGEM-T mini-prep DNA (see Fig. 4.3.3a for a photograph of the PCR-amplified bands, and Fig. 4.3.3b for an interpretation of the results). Clones were obtained of the four possible splice variants: H2a, H2b and H2c, plus the H2 splice variant – unnamed in the literature - that lacks the 57 bp but not the 15 bp insert. Another putative splice variant, which lacks 174 bp instead of 57 bp (and unmentioned in the literature), was also identified (clone 1 in Fig. 4.3.3.).



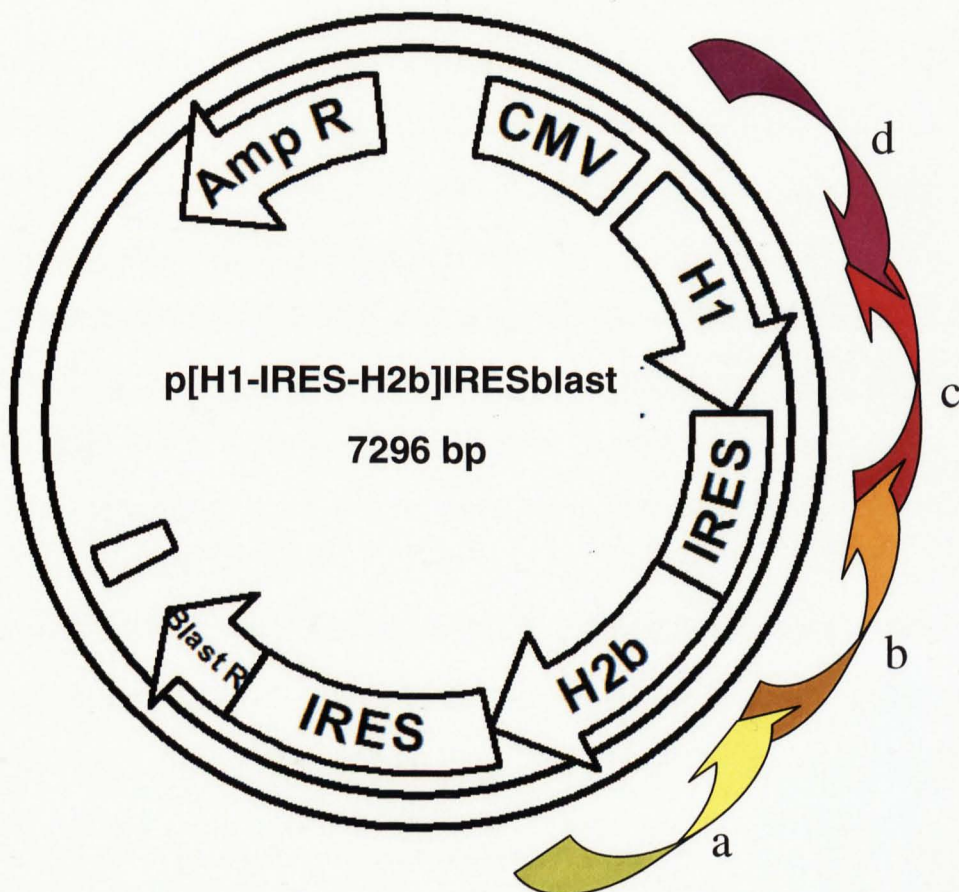


Fig. 4.3.1. The design of the intended vector and the primers used for verification by sequencing

Primers:		Primer sequence (5' end):
5'	3'	
		cgcttttgagagggagtactc
		gaggagctccatctggcaggccac
		caactcacaacgtggcactg
		cgactcactataggagacc

Fig. 4.3.2. (Facing page) Asialoglycoprotein receptor H2 subunit sequence, showing the different possible splice variants and primers used for their identification.

The 57 bp and 15 bp inserts are highlighted in yellow and green respectively. The primers used for identification of the 57 bp and 15 bp insert are shown as red and blue arrows respectively. The conserved splice donor (**GT**) and splice acceptor (**AG**) sites present in the intron borders are shown in bold type. The 174 bp insert spliced out in one clone (underlined) is referred to in the discussion. The transmembrane domain is encoded by bases 372 to 435.



1 CTGCGGGAGC CAGGCGTCCG CTCTCCACAC CTTTCACAGA TCCTACCCAG  
51 GCCACCACCC CGAACCCCTC CGACCCTGCG TTCCATCCCA CCATCAACCT  
101 CCACGTGAAT CTGCACCTCT GCCCCAGCCC CAGCCCTCAG AGCAACCTCA  
151 GCCCAGCCCC GCCCAGCTCC AGCTCCAGCT CCAGCCCCGGG Start  
201 GCCAAGGACT TTCAAGATAT CCAGCAGCTG AGCTCGGAGG AAAATGACCA  
251 TCCTTTCCAT CAAGGTGAGG GGCCAGGCAC TCGCAGGCTG AATCCCAGGA  
301 GAGGAAATCC ATTTTGTAAA GGGCCACCTC CTGCCCAGCC CCTGGCACAG  
351 CGTCTCTGCT CCATGGTCTG CTTCAGTCTG CTTGCCCTGA GCTTCAACAT  
401 CCTGCTGCTG GTGGTCATCT GTGTGACTGG GTCCCAAGT GAGGGTCACA  
451 GAGGTGCACA GCTGCAAGCC GAGCTGCGGA GCCTGAAGGA AGCTTTCAGC  
501 AACTTCTCCT CGAGCACCTT GACGGAGGTC CAGGCAATCA GCACCCACGG  
551 AGGCAGCGTG GGTGACAAGA TCACATCCCT AGGAGCCAAG CTGGAGAAAC  
601 AGCAGCAGGA CCTGAAAGCA GATCACGATG CCCTGCTCTT CCATCTGAAG  
651 CACTTCCCCG TGGACCTGCG CTTCGTGGCC TGCCAGATGG AGCTCCTCCA  
701 CAGCAACGGC TCCCAAAGGA CCTGCTGCCC CGTCAACTGG GTGGAGCACC  
751 AAGGCAGCTG CTA CTGTTT TCTACTCCG GGAAGGCCTG GGCTGAGGCG  
801 GAGAAGTACT GCCAGCTGGA GAACGCACAC CTGGTGGTCA TCAACTCCTG  
851 GGAGGAGCAG AAATTTCATTG TACAACACAC GAACCCCTTC AATACCTGGA  
901 TAGGTCTCAC GGACAGTGAT GGCTCTTGGA AATGGGTGGA TGGCACAGAC  
951 TATAGGCACA ACTACAAGAA CTGGGCTGTC ACTCAGCCAG ATAATTGGCA  
1001 CGGGCACGAG CTGGGTGGAA GTGAAGACTG TGTGAAAGTC CAGCCGGATG  
1051 GCCGGCTGGA ACGATGACTT CTGCCTGCAG GTGTACCGCT GGGTGTGTGA  
1101 GAAAAGGCGG AATGCCACCG GCGAGGTGGC CTGACCCAG CACACCTCTG  
1151 GCTAACCCAT ACCCCACACC TGCCAGCTC TGGCTTCTCT GTTGAGGATT  
1201 TTGAGGAAAG GAAGAAACAC TGAGACAGGG GTATGGGGAA GAGCTGAGCA  
1251 AAGAGAGAAA GGAGGTAGTT TAAGAGTCCC TGACCCTGGA GGA CTGAGAT  
1301 CCCACCTCCT TCTGTAATTC ATTGTAATTA TTATAATCGT CAGCCTCTTC  
1351 AATGGCGTAG GAAAGAAGAA ACAAATGCTT GA

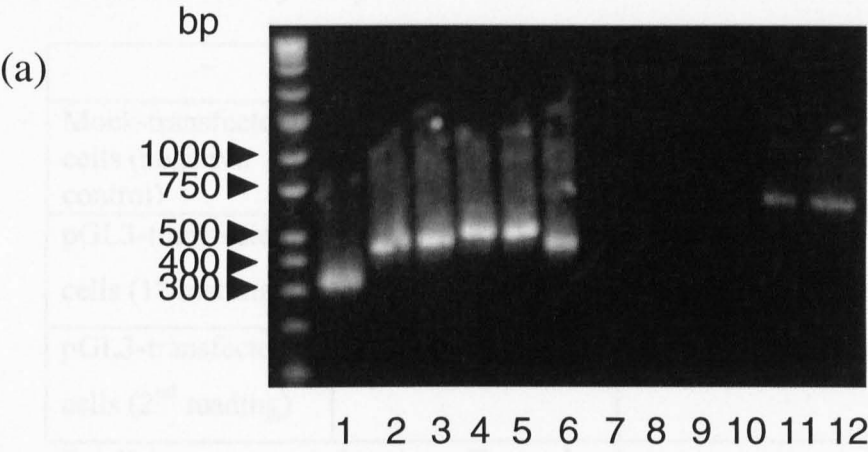
Fig. 4.3.3. (Facing page) Splice variant identification of ASGP-R H2 clones.

(a) Analytical PCR of ASGP-R-H2 cDNA clones. Ethidium Bromide-stained 1% agarose gel loaded as follows:

-Lanes 1-6; Clones 1-6 PCR amplified with primers 57-5' & 57-3' to establish whether they contain the 57 bp insert (slightly slower migration indicates presence of insert).

-Lanes 7-12; Clones 1-6 PCR amplified with primers 15-5' and 15-3' to establish whether they contain the 15 bp insert (presence of ~700 bp amplified fragment indicates presence of insert).

(b) Table summarising the results obtained from the Ethidium Bromide-stained gel. A clone missing the 57 bp insert but containing the 15 bp insert is indicated by "?" (it is not named in the literature).



(b)

Clone	57 bp insert	15 bp insert	Identity
1	- (174 bp missing)	-	?
2	-	-	H2c
3	-	-	H2c
4	+	-	H2b
5	+	+	H2a
6	-	+	?

#### **4.3.3. Screening of transfection reagents**

The AS-E2 cell line was transfected with a luciferase-based reporter plasmid (pGL3, Promega) using a range of transfection reagents (FuGENE 6 (Roche Diagnostics), GeneJuice (Novagen) and SuperFect (Qiagen)) and screening was carried out by luciferase activity assay.

	<b>FuGENE 6</b>	<b>GeneJuice</b>	<b>SuperFect</b>
Mock-transfected cells (negative control)	417	394	402
pGL3-transfected cells (1 <sup>st</sup> reading)	3092	9759	1285
pGL3-transfected cells (2 <sup>nd</sup> reading)	2962	11887	991

Readings were carried out on a Turner luminometer and are in arbitrary units.

Use of the GeneJuice transfection reagent resulted in at least a threefold increase in luciferase activity over the other two reagents screened, indicating better efficiency in transfections and thus increasing the chance of obtaining a stable transfection event.

GeneJuice was therefore used in all subsequent transfections.

#### **4.3.4. Expression of the ASGP-R in AS-E2 cells**

AS-E2 cells were transfected with the p[H1-IRES-H2b]IRESblast vector as described in Section 2.2.3.1. and selected with blasticidin (10 µg/ml). After 10 passages a polyclonal cell line designated AS-E2(H1-IRES-H2b) was derived.

Cells were harvested by centrifugation, dissolved in SDS-loading buffer and loaded on an SDS-PAGE gel (Sections 2.3.1, 2.3.3. and 2.3.4.) along with untransfected cells which were used as a negative control and Hep-G2 cells as a positive control (see Fig. 4.3.4.).



Fig. 4.3.4. (Facing page) SDS-PAGE/Western blot of ASGP-R-H1 expressed recombinantly in AS-E2 cells and endogenously in Hep-G2 cells, using an anti-ASGP-R antibody. A Western blot for tubulin is also provided, to give an indication of protein loading. Triton X-100 lysates were made, normalised for protein content by BCA assay, run on SDS-PAGE, transferred to membrane and Western blotted with the relevant antibody (following protocol in Sections 2.3.1-5.).

(a) Probed with anti-ASGP-R-H1 (Calbiochem).

-Lane 1; untransfected AS-E2 cell lysate.

-Lane 2; lysate from AS-E2 cells stably transfected with cDNA encoding ASGP-R-H1 and ASGP-R-H2b.

-Lane 3; Hep-G2 lysate.

(b) Probed with anti-tubulin (Santa Cruz Biotechnology).

-Lane 1; untransfected AS-E2 cell lysate.

-Lane 2; lysate from AS-E2 cells stably transfected with cDNA encoding ASGP-R-H1 and ASGP-R-H2b.

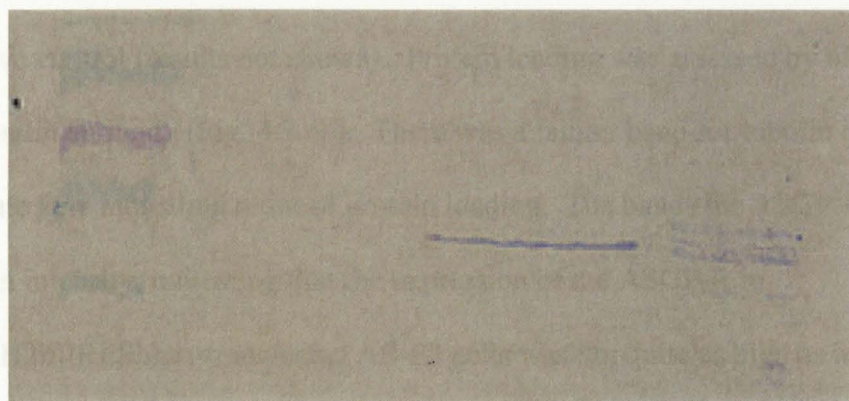
-Lane 3; Hep-G2 lysate.

(a) kDa

64.2 ►

48.8 ►

37.1 ►



1

2

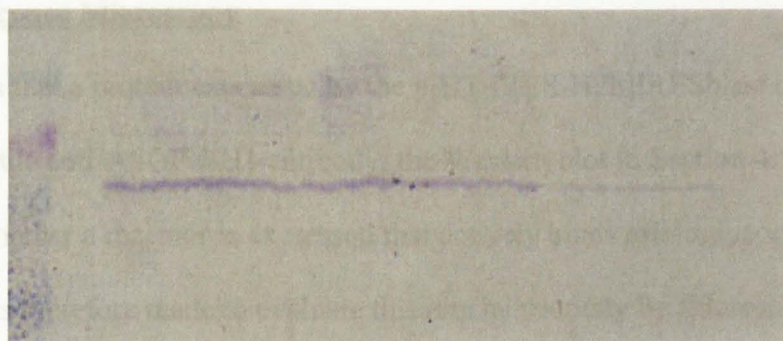
3

(b) kDa

64.2 ►

48.8 ►

37.1 ►



1

2

3



Expression of ASGP-R-H1 was detected by Western blotting with mouse monoclonal antibodies that recognise ASGP-R-H1 (Calbiochem). A protein of  $M_r = 46$  kDa, the size expected for the asialoglycoprotein receptor, was expressed by stably transfected cells but was absent from untransfected cells (Fig. 4.3.4a). Separate antibodies against ASGP-RH1 and ASGP-RH2 from Santa Cruz were also used in a Western blot but these were unable to differentiate between untransfected AS-E2 cells and the Hep-G2 lysate positive control (results not shown). Protein loading was assessed by blotting with anti-tubulin antibody (Fig. 4.3.4b). There was a fainter band for tubulin in the Hep-G2 lysate lane indicating reduced protein loading. The bands for ASGP-R-H1 were equal in intensity, indicating that the expression of the ASGP-R in p[H1-IRES-H2b]IRESblast-transfected AS-E2 cells was not quite as high as in the Hep-G2 cell line.

#### **4.3.5. Fluorescence Microscopy**

While it shows that a protein expressed by the p[H1-IRES-H2b]IRESblast cells is recognised by the anti-ASGP-RH1 antibody, the Western blot in Section 4.3.4. does not indicate whether a receptor is expressed that actively binds asialoglycoproteins. An attempt was therefore made to evaluate this crucial property by fluorescence microscopy. An asialoglycoprotein frequently used in such ASGP-R studies, alpha-1-acid glycoprotein, was prepared by sialidase treatment (as in Section 2.3.10.) and conjugated to the fluorochrome Alexa Fluor 488 (a low molecular weight green dye with absorption/emission spectra similar to those of fluorescein). AS-E2 cells transfected with empty vector (pIRESblast) and with p[H1-IRES-H2b]IRESblast were incubated with the above reagent prior to their actin cytoskeleton and nuclei being stained (by Hoechst and Phalloidin 594 respectively) to aid in cell localisation

(Section 2.3.13.). It was possible to distinguish between the two cell lines (see Fig. 4.3.5.), with green areas (indicating asialoglycoprotein binding) being visible in the p[H1-IRES-H2b]IRESblast-transfected cells but not in the empty vector-transfected cells, confirming that the transfected cell line expressed an asialoglycoprotein-binding receptor.

Fig. 4.3.5. (Facing page) Expression of functional ASGP-R: binding of fluorophore-labelled asialo- $\alpha$ -1-acid glycoprotein.

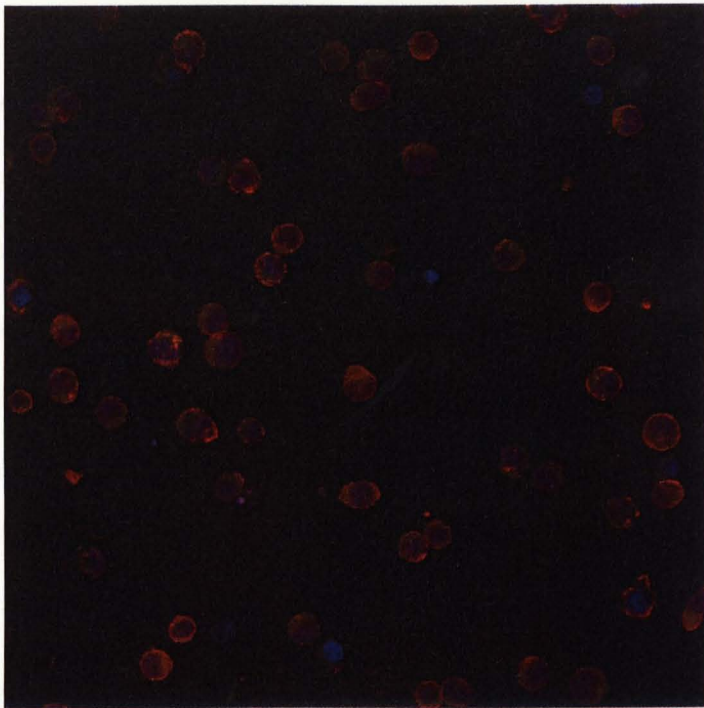
Fluorescence images of cells stained with Phalloidin-574 (red), Hoechst (blue) and Alexa-Fluor conjugated-asialo- $\alpha$ -1-acid glycoprotein (green). See Section 2.3.13. for explanation and protocol.

(a) AS-E2 cells stably transfected with pIRESblast empty vector.

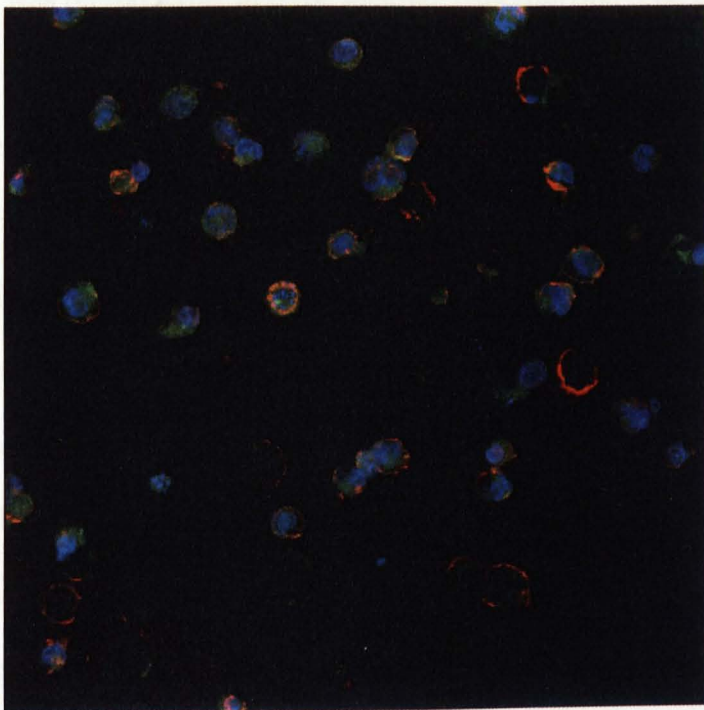
(b) AS-E2 cells stably transfected with pIRESblast[H1-IRES-H2b], a vector encoding the two asialoglycoprotein subunits.

Cells are approximately 20  $\mu$ M in diameter.

(a)



(b)



#### **4.3.6. Bioassays**

##### **4.3.6.1. Evaluating sensitivity to desialylation in the ASGP-R-transfected AS-E2 cell line**

The empty vector- and p[H1-IRES-H2b]IRESblast-transfected AS-E2 polyclonal cell lines were used in a bioassay comparing the intact Epo ( $t=0$  min) and the most desialylated Epo sample ( $t=1800$  min) from Section 3.3.1. As can be seen in Fig. 4.3.6., the two cell lines responded differently to the two forms of Epo. In the empty vector-transfected AS-E2 cells, desialylated Epo ( $t=1800$  min) produced a greater response than did intact Epo (sample  $t=0$  min), having 1.239 times its potency (Table 4.3.1.). By contrast, desialylated Epo ( $t=1800$  min) produced a lower response in the p[H1-IRES-H2b]IRESblast-transfected AS-E2 cells compared with intact Epo (sample  $t=0$  min), registering only 0.705 times its potency. The effect seen in Fig. 4.3.6. was reproducible in three separate experiments. The H1H2b-transfected cells showed the difference in the 0.05-0.2 nM range of Epo concentrations. Differences between the preparations were lost at higher than saturating concentrations.

The experiment was repeated, but with the extensively desialylated Epo sample  $t=1800$  min replaced by the less desialylated  $t=480$  min and  $t=20$  min Epo samples in order to determine at what point the assay no longer distinguished between two differentially sialylated Epo samples. The mean pI of Epo sample  $t=480$  min (5.83) is approximately midway between that of Epo samples  $t=1800$  min (6.65) and  $t=0$  min (4.21). Fig. 4.3.7. shows that, compared to the empty-vector-transfected cell line, the p[H1-IRES-H2b]IRESblast assay still produces statistically significant differentiation (as evidenced by the non-overlapping 95% confidence intervals in Table 4.3.2.)

Fig. 4.3.6. (Facing page) Proliferative response of empty vector-transfected AS-E2 cells and AS-E2 cells stably transfected with the asialoglycoprotein receptor H1 and H2b subunits to both intact and the most extensively desialylated Epo (samples  $t=0$  min and  $t=1800$  min from Chapter 3).

(a) Empty vector (pIRESblast)-transfected AS-E2 cells.

(b) AS-E2 cells transfected with the ASGP-R-H1- and -H2b-encoding vector (pIRESblast[H1-IRES-H2b]).

Sample  $t=0$  min (■) and sample  $t=1800$  min (●). Data are presented as the mean  $\pm$  s.e.m. of triplicated samples (where an error bar is not shown, it lies within the dimensions of the symbol). The effect seen was reproducible and achieved in 3 separate experiments.

Cell Line	Potency of sample $t=1800$ min relative to sample $t=0$ min	95% Confidence interval
AS-E2 cells transfected with empty vector (pIRESblast)	1.239	1.137 – 1.353
AS-E2 cells transfected with the ASGP-R H1- and H2b-encoding vector (pIRESblast[H1-IRES-H2b]).	0.705	0.653 – 0.759

Table 4.3.1. Potencies of desialylated Epo (sample  $t=1800$  min) relative to intact Epo (sample  $t=0$  min) as calculated by the RANDOM program (see Section 2.2.4.1. for more detail).

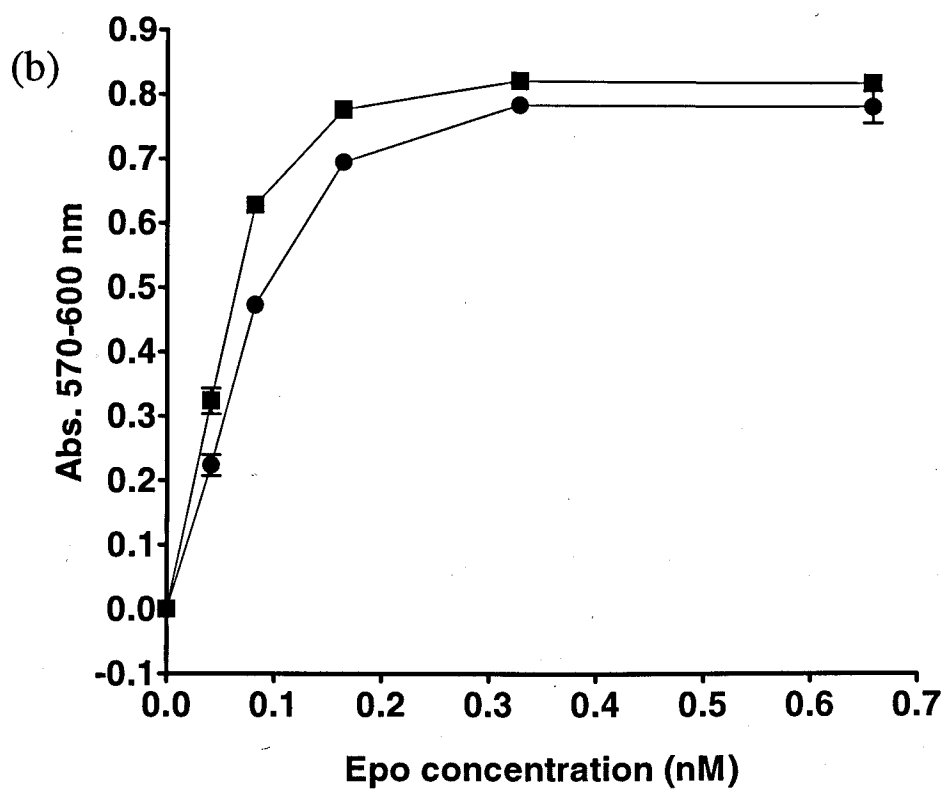
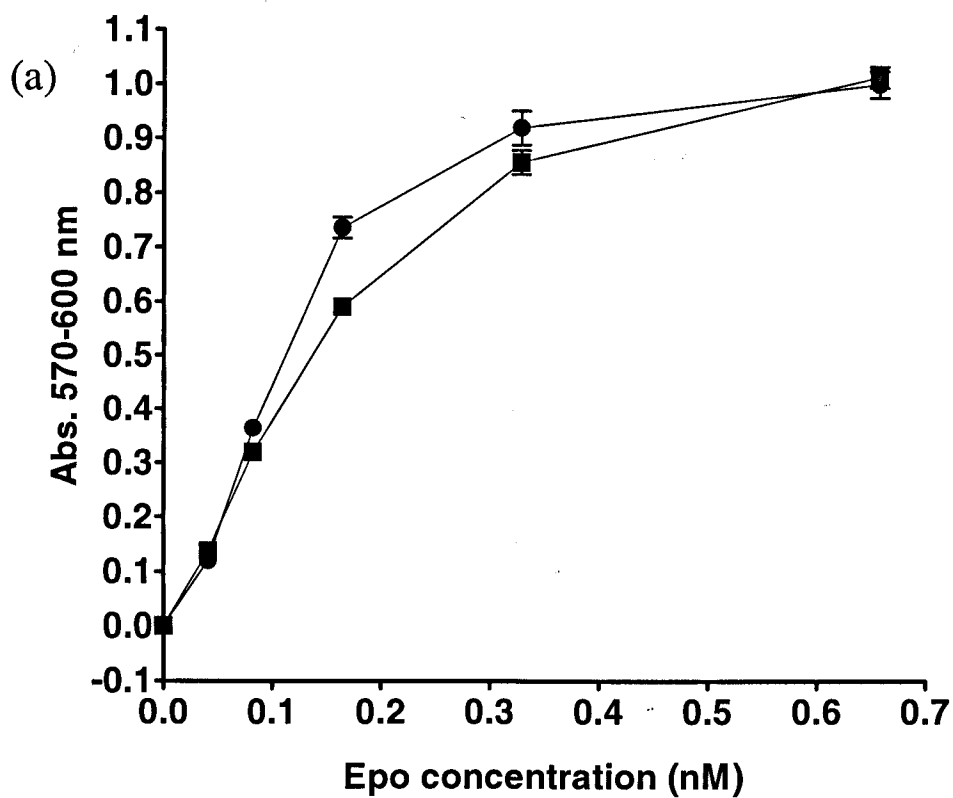


Fig. 4.3.7. (Facing page) Proliferative response of empty vector-transfected AS-E2 cells and AS-E2 cells stably transfected with the asialoglycoprotein receptor H1 and H2b subunits to both intact and mid-desialylated Epo (samples t=0 min and t=480 min from Chapter 3).

(a) Empty vector (pIRESblast)-transfected AS-E2 cells.

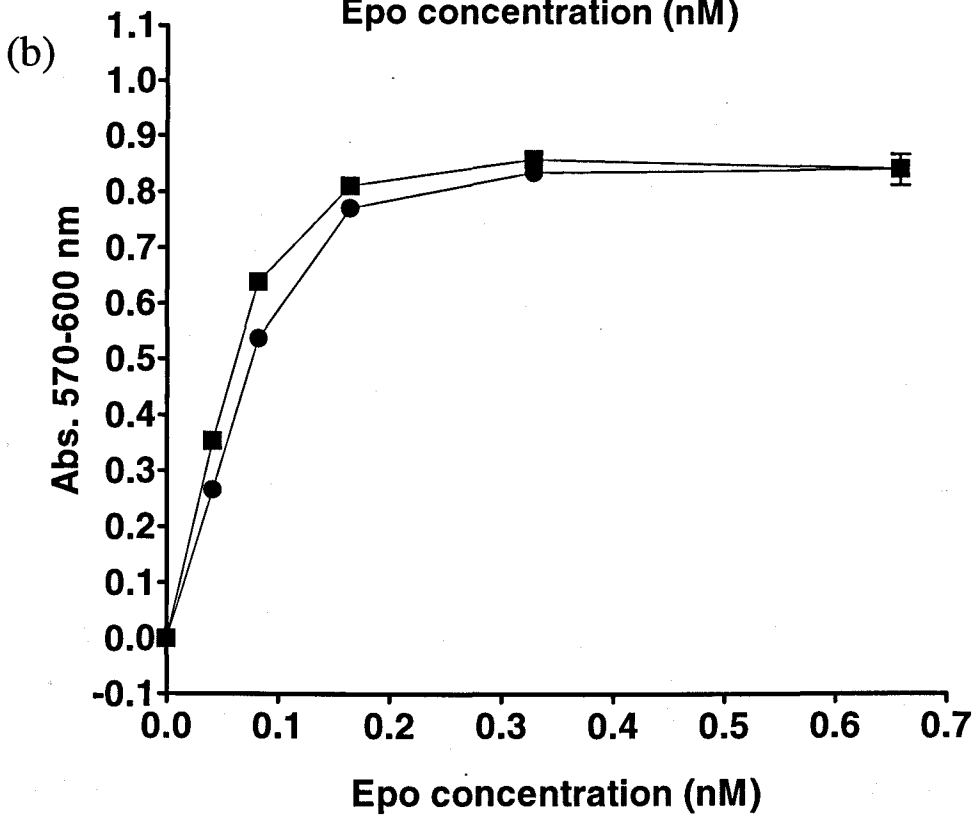
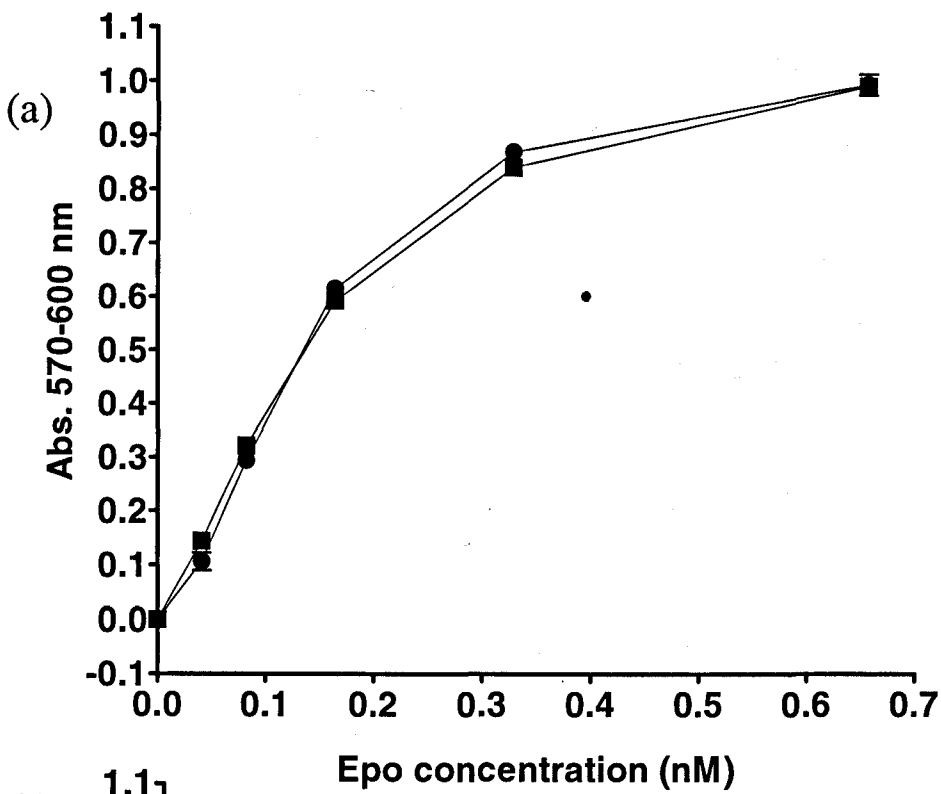
(b) AS-E2 cells transfected with the ASGP-R-H1- and -H2b-encoding vector (pIRESblast[H1-IRES-H2b]).

Sample t=0 min (■) and sample t=480 min (●). Data are presented as the mean  $\pm$  s.e.m. of triplicated samples (where an error bar is not shown, it lies within the dimensions of the symbol).

Cell Line	Potency of sample t=480 min relative to sample t=0 min	95% Confidence interval
AS-E2 cells transfected with empty vector (pIRESblast)	0.961	0.917 – 1.006
AS-E2 cells transfected with the ASGP-R H1- and H2b-encoding vector (pIRESblast[H1-IRES-H2b]).	0.802	0.763 – 0.842

Table 4.3.2. Potencies of desialylated Epo (sample t=480 min) relative to intact Epo (sample t=0 min) as calculated by the RANDOM program.





between Epo samples  $t=480$  min and  $t=0$  min. The difference between the two was less marked, however, in that sample  $t=480$  min displayed approximately the same level activity as that of intact Epo sample  $t=0$  min in the empty-vector-transfected cell assay (96%) but was of a lower activity in the p[H1-IRES-H2b]IRESblast assay (80%).

The assay could not distinguish between the intact and slightly desialylated Epo samples (Epo samples  $t=0$  min and  $t=20$  min respectively), with the  $t=20$  min Epo sample having the same potency in relation to intact Epo (sample  $t=0$  min), irrespective of the cell line in which it was being assayed (in both cases it was equivalent to 87% of  $t=0$  min, Fig. 4.3.8).

The maximum proliferation of the two AS-E2 cell lines (empty vector- and ASGP-R-transfected) was unaffected by the transfection of AS-E2 cells with ASGP-R-H1 and ASGP-R-H2b since presumably once the ASGP-R binding sites were fully occupied the rest of the Epo could simply continue binding Epo-R, which has a very high binding affinity (the ASGP-R has a 10 nM dissociation constant for asialo- $\alpha$ -1-acid glycoprotein (Bider *et al*, 1995) while that of the low-affinity Epo receptor is around ten times lower at 0.8-1.3nM (Dong *et al*, 1992). Neither the receptor nor the signal pathways were affected in any way so there was no difference in maximal proliferation.

#### **4.3.6.2. The effect of asialo- $\alpha$ -1-acid glycoprotein**

In order to show that the decoy receptor effect observed in Section 4.3.4.1. was specifically attributable to the action of the ASGP-R, the assay was performed twice more in the presence of either an excess of sialidase-treated (and therefore asialo-)  $\alpha$ -1-acid glycoprotein (Sigma, UK) or fully sialylated  $\alpha$ -1-acid

Fig. 4.3.8. (Facing page) Proliferative response of empty vector-transfected AS-E2 cells and AS-E2 cells stably transfected with the asialoglycoprotein receptor H1 and H2b subunits to both intact and slightly desialylated Epo (samples  $t=0$  min and  $t=20$  min from Chapter 3).

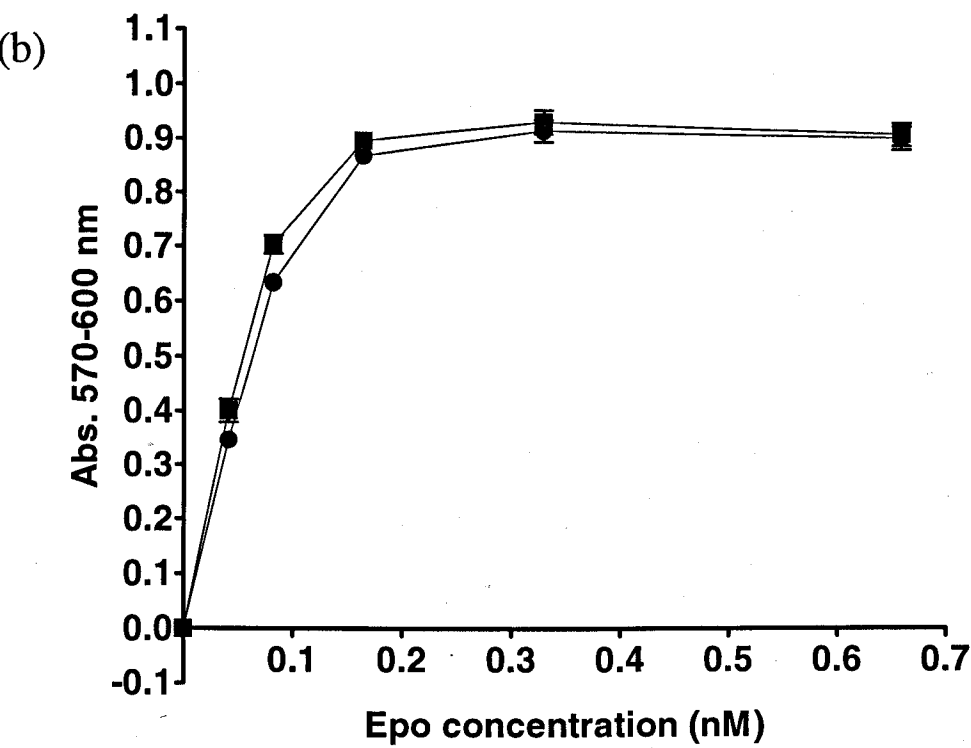
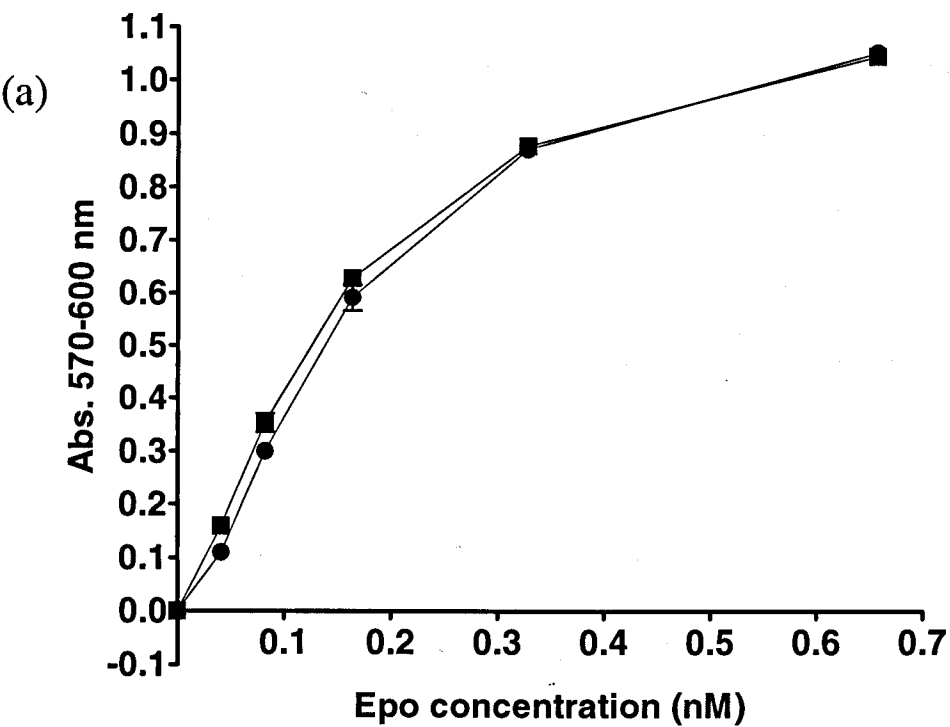
(a) Empty vector (pIRESblast)-transfected AS-E2 cells.

(b) AS-E2 cells transfected with the ASGP-R-H1- and -H2b-encoding vector (pIRESblast[H1-IRES-H2b]).

Sample  $t=0$  min (■) and sample  $t=20$  min (●). Data are presented as the mean  $\pm$  s.e.m. of triplicated samples (where an error bar is not shown, it lies within the dimensions of the symbol).

Cell Line	Potency of sample $t=20$ min relative to sample $t=0$ min	95% Confidence interval
AS-E2 cells transfected with empty vector (pIRESblast)	0.873	0.805 – 0.946
AS-E2 cells transfected with the ASGP-R H1- and H2b-encoding vector (pIRESblast[H1-IRES-H2b]).	0.871	0.809 – 0.936

Table 4.3.3. Potencies of desialylated Epo (sample  $t=20$  min) relative to intact Epo (sample  $t=0$  min) as calculated by the RANDOM program.

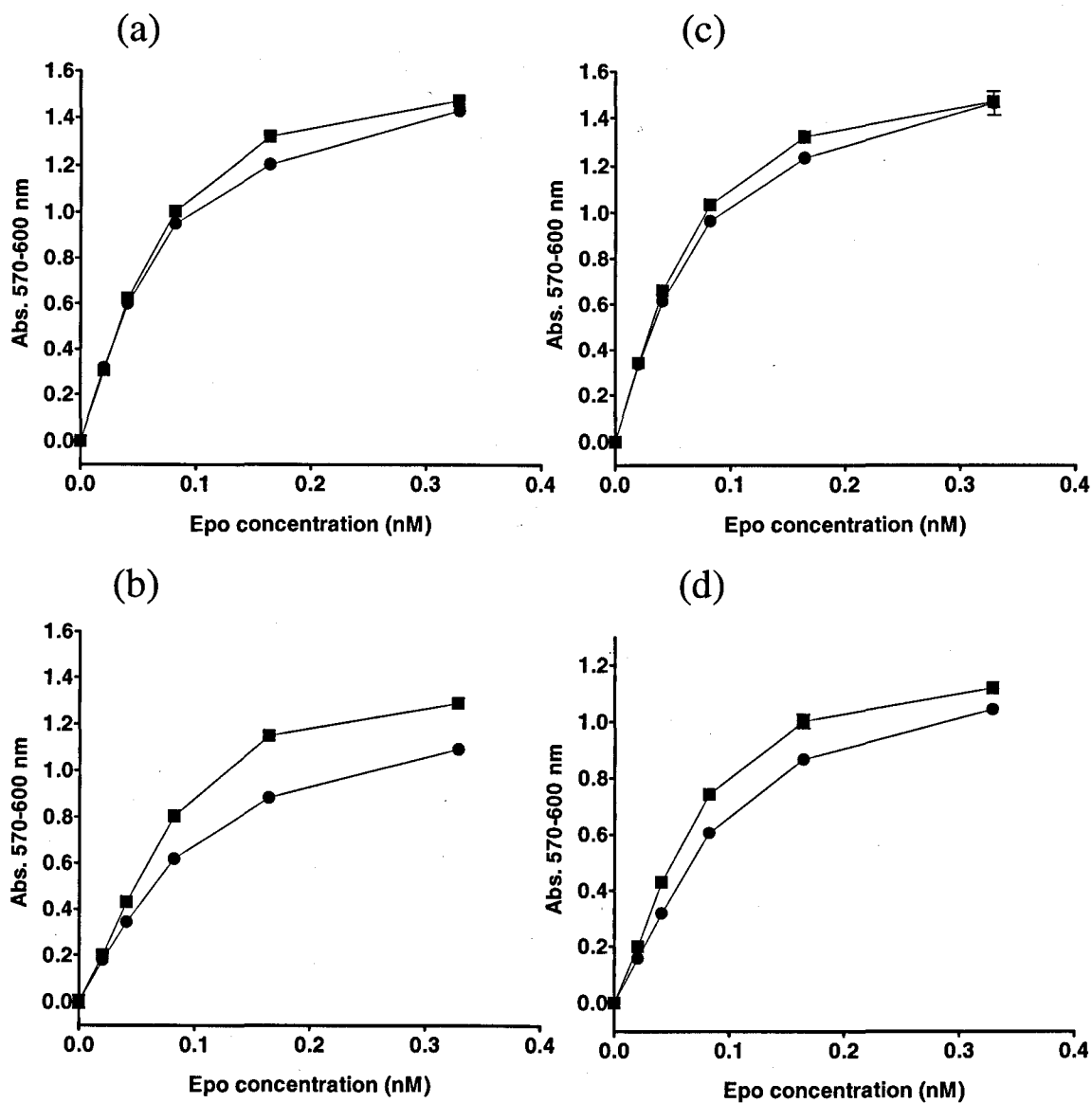


glycoprotein. As stated above (Section 4.3.3.), asialo-alpha-1-acid glycoprotein is frequently used in ASGPR-binding studies and is therefore ideal for competitively binding this receptor. Intact alpha-1-acid glycoprotein was used as a control. The results are shown in Fig. 4.3.9. In the assays represented in Figs. 4.3.9.a and b, the medium included an excess of fully sialylated alpha-1-acid glycoprotein, a protein not expected to affect cell proliferation. In the empty vector-transfected AS-E2 assay, extensively desialylated Epo (sample t=1800 min) had 86% of the potency of intact Epo (sample t=0 min). In the pIRESblast[H1-IRES-H2b]-transfected cell assay, extensively desialylated Epo (sample t=1800 min) had 62.5% of the potency of intact Epo (sample t=0 min). These two assays were repeated (Figs. 4.3.9c and d), this time with asialo-alpha-1-acid glycoprotein in the medium. The resulting excess of exposed galactose residues was expected to saturate all the ASGP-R galactose-binding sites, cancel out any function this receptor performs in the assay and effect a return to the dose response curve recorded in the empty vector-transfected AS-E2 cell assay. In the empty vector-transfected AS-E2 cell assay in the presence of asialo-alpha-1-acid glycoprotein, sample t=1800 min was as active relative to sample t=0 min as in the presence of intact alpha-1-acid glycoprotein (87.7%). In the pIRESblast[H1-IRES-H2b]-transfected cell assay, the activity of desialylated Epo sample t=1800 min in relation to intact Epo sample t=0 min rose to 71.6%. The same effect was observed when using an excess concentration of the ASGP-R-inhibiting sugar lactose (100 mM) but not when using the same concentration of glucose, which does not bind the ASGP-R (results not shown). In conclusion, lower rates of proliferation were observed in the pIRESblast[H1-IRES-H2b]-transfected cell line when stimulated with desialylated compared to intact Epo. The difference was reduced when the ASGP-R ligand

Fig. 4.3.9. (Facing page) Proliferative response of empty vector-transfected AS-E2 cells and AS-E2 cells stably transfected with the asialoglycoprotein receptor H1 and H2b subunits to both intact and desialylated Epo (samples  $t=0$  min and  $t=1800$  min) in the presence of intact alpha-1-acid glycoprotein (AAGP) or asialo-AAGP.

- (a) Empty vector (pIRESblast)-transfected AS-E2 cells in the presence of intact AAGP.
- (b) AS-E2 cells transfected with the ASGP-R H1- and H2b-encoding vector (pIRESblast[H1-IRES-H2b]) in the presence of intact AAGP.
- (c) Empty vector-transfected AS-E2 cells (pIRESblast) in the presence of asialo-AAGP.
- (d) AS-E2 cells transfected with the ASGP-R H1- and H2b-encoding vector (pIRESblast[H1-IRES-H2b]) in the presence of asialo-AAGP.

In all graphs, sample  $t=0$  min (■) and sample  $t=1800$  min (●). Data are presented as the mean  $\pm$  s.e.m. of triplicated samples (where an error bar is not shown, it lies within the dimensions of the symbol). A table is provided summarising the potencies of sample  $t=1800$  min relative to sample  $t=0$  min in each of the four assays (calculated using the RANDOM program).



Graph	Potency of sample t=1800 min relative to sample t=0 min	95% Confidence interval
(a) Empty vector/intact AAGP	0.864	0.842 – 0.886
(b) Empty vector/asialo- AAGP	0.625	0.595 – 0.655
(c) H1H2b/intact AAGP	0.877	0.813 – 0.945
(d) H1H2b/asialo-AAGP	0.716	0.675 – 0.759

asialo- $\alpha$ -1-acid glycoprotein was included in the assay, with proliferation levels for desialylated Epo returning to those obtained for intact Epo. This showed that the ASGP-R was responsible for the lower rates of proliferation.

## **4.4. Discussion**

### **4.4.1. Optimisation of stable transfection protocol**

The technique of obtaining protein-of-interest-expressing stable transfectants is acknowledged as being a potential rate-limiting step in expression studies. Two major amendments to the vectors used were necessary before a reliable method of obtaining recombinant protein-expressing stably transfected clonal cell lines could be achieved.

The final vector used had the following features:

- (1) Constitutive, non-amplified expression of the gene of interest;
- (2) Blasticidin selection;
- (3) IRES technology.

Preliminary attempts were made at stably transfecting AS-E2 cells using the pBudCE4.1 vector from Invitrogen, which uses the Zeocin selectable marker and has two unlinked expression cassettes: one driven from a CMV promoter, the other from an EF-1 $\alpha$  promoter. No stable cell lines resulted as Zeocin did not kill cells quickly or efficiently enough. The Zeocin resistance gene was therefore replaced with a blasticidin resistance gene from the pcDNA 6.2-DEST Gateway vector (Invitrogen). Blasticidin is reputed to be the fastest and most potent selection reagent available (Izumi *et al*, 1991). It is a peptidyl nucleoside antibiotic derived from *Streptomyces griseochromogenes*, and works by specifically inhibiting protein synthesis, more



specifically peptide-bond formation in the ribosomal complex. Blasticidin can be used to select for transfected cells carrying bsr (Endo *et al*, 1988) or BSD (Kimura *et al*, 1994) resistance genes (the BSD gene from *Aspergillus terreus* in the case of pcDNA 6.2-DEST Gateway). Switching to this new version of the vector made it possible to generate antibiotic-resistant clonal cell lines, six of which were screened by Western blot using the anti-ASGP-R antibody, giving a negative result in each case, possibly because of deletion or inactivation of the ASGP-R genes following integration. Even if such stable cell lines expressing the ASGP-R proteins had arisen, expression levels can be unpredictable and decrease during extended cultivations since there is no selection pressure ensuring transcription (Fussenegger *et al*, 1998). These drawbacks prompted a second change in vector design – one that exploited the benefits of the Internal Ribosome Entry Site (IRES).

#### **4.4.2. The Internal Ribosome Entry Site (IRES)**

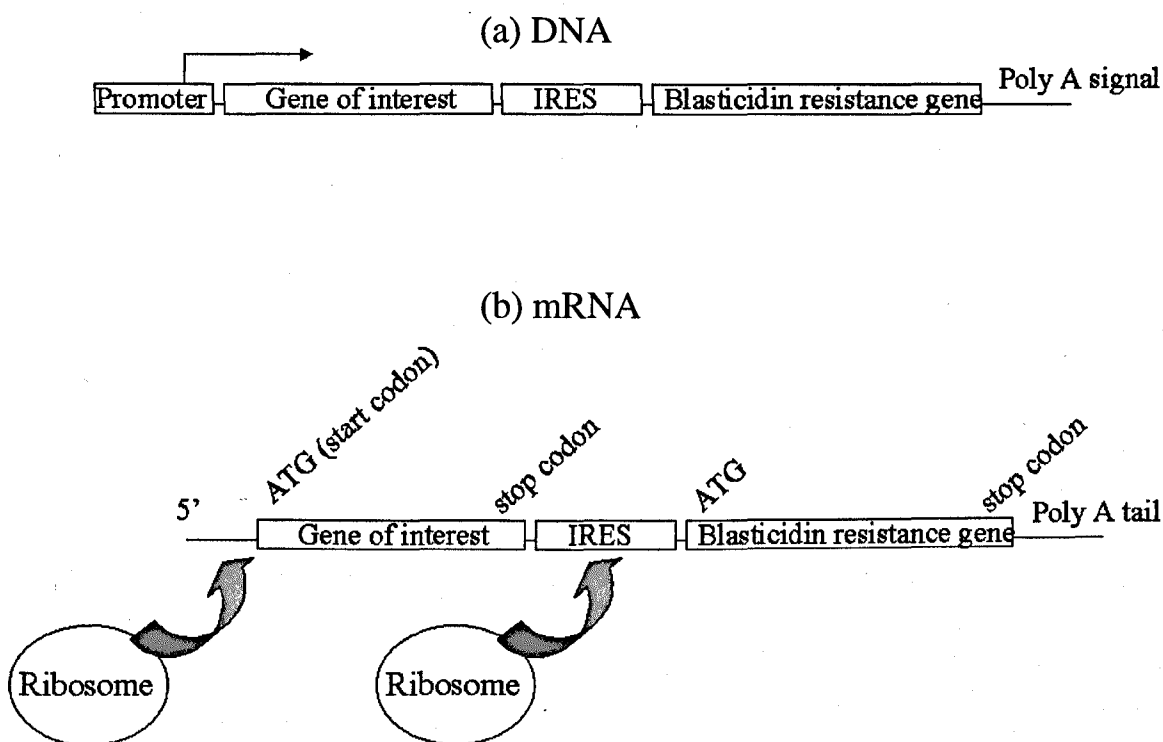
The integration of transfected DNA into mammalian genomes is unpredictable and occurs by non-homologous recombination (Fussenegger *et al*, 1999). Selecting cell lines in which all cells express the gene of interest at reasonable levels is a laborious process, further hampered by the fact that expression can be adversely affected by the plasmid becoming integrated in a transcriptionally silent region of the genome or by the fact that for integration into the genome to occur the plasmid must undergo recombination with the chromosome, an event that could cause disruption to expression if it takes place within the gene of interest. If the antibiotic resistance gene and the gene of interest are transcribed from different promoters (i.e. are unlinked) then antibiotic-resistant cell lines may be produced, but there is no guarantee that the gene of interest will also be expressed (Fussenegger *et al*, 1998). The chances of

success have been put at little better than 30%, with the authors of the paper (Rees *et al*, 1996) finding just one recombinant protein expressor among 19 clonal cell lines screened when using pcDNA3.1. Using an IRES-containing vector is one way of overcoming these problems. The IRES, first discovered in picornaviruses (Jang *et al*, 1988; Jang *et al*, 1989), is a highly structured region of RNA that allows translation of two proteins from a single mRNA, termed di-cistronic translation. In the standard model of transcription, ribosomes enter at the 5' cap structure and reach the initiation codon (ATG), whereupon they begin translation. In the case of the IRES vector, transcription from both the antibiotic-resistance gene and the gene of interest is driven from the same promoter (see Fig. 4.4.1. for a diagram describing how the IRES functions). In essence, the IRES provides a built-in guarantee that any resistant cells will produce mRNA for both subunits. An IRES sequence was inserted between the two genes of interest to ensure expression of both the H1 and H2b subunits. This strategy has been used by Fussenegger *et al* (1998;1997). They made a quattrocistronic mammalian expression vector by ligating the cassette "MCSI-IRESI-MCSII-IRESII-MCSIII" (where MCS stands for multiple cloning site) into the pIRESneo vector (Clontech). They reasoned that, following selection with neomycin, the IRES sequences ensure that all proteins of interest are expressed over time.

Rees *et al* (1996) reported that adjustable expression was possible using the pIRESneo vector by gradually increasing the concentration of neomycin in the medium. This was not observed when increasing the concentration of blasticidin in the medium of cells transfected with the pIRESblast vector generated in Section 2.2.3.2.

Fig. 4.4.1. Schematic representation of the IRES cassette at (a) DNA and (b) mRNA levels.

Ribosomes enter the mRNA at the 5' end, translating the gene of interest (as in the standard model of transcription). They also enter at the IRES, translating the blasticidin resistance gene. This ensures that if the blasticidin resistance gene is being expressed, the mRNA encoding the gene of interest must also have been synthesised (the former does not have its own promoter and it is therefore not possible for its mRNA alone to be transcribed).



The IRES sequence imposes the constraint that the gene inserted after the IRES will be expressed at lower levels. H1 on the surface of Hep-G2 exceeds H2 by a factor of about three (Henis *et al*, 1990) and was therefore chosen for ligation into the higher expressing first multiple cloning site.

#### **4.4.3. Putative new splice variant**

The PCR-amplified band from clone 1 in Fig.4.3.3. migrated faster than the other PCR bands, suggesting the possible presence of a new splice variant of H2. Sequencing revealed a 174 bp deletion which, since it is still in frame, would not create a nonsense mutation (see Fig. 4.3.2.). It is not clear whether the region spliced out, which corresponds to the transmembrane domain of the ASGP-R-H2 subunit, is an artefact created by an indiscriminate splicing event occurring between the consensus splice sites of two different introns or a new splice variant encoding another soluble form in addition to those created by the 15 bp mini-exon (Lederkremer & Lodish, 1991). It has been suggested that the 15 bp insert may almost invariably end up being spliced out and that a stage is being amplified that has not yet been completely processed. Lederkremer *et al* (1991) reported that 70% of 3T3 cells infected with virion-packaged H2a expressed the H2b subunit.

#### **4.4.4. Soluble receptors**

Heaney and Golde (Heaney & Golde, 1996) describe soluble receptors as being produced either by proteolytic cleavage of an expressed membrane-bound receptor to produce a soluble form, or by an alternative mRNA splice resulting in a transcript missing the transmembrane-encoding region. The role of soluble receptors has not been fully established, although the fact they are so abundant suggests it may be

important. Possibilities include: inhibition by extracellular ligand sequestration (thereby preventing binding to the membrane-bound receptor and consequent signal transduction), ligand stabilisation, increase in sensitivity to ligand by enhanced delivery to the membrane-bound receptor, and down-regulation of the membrane-bound receptor (by transformation into a soluble non-signal transducing form) (Heaney & Golde, 1996).

The discovery of what is potentially a new soluble variant raises the question of whether such a receptor could be exploited, for example for use in inhibition assays. This would not be a viable alternative, however, because, as mentioned in Section 1.15., the soluble form does not achieve the same high-affinity binding as does the membrane-bound form (Baenziger & Fiete, 1980). Moreover, since plant lectins did not act as competitive antagonists (Section 3.3.1.7.) it is doubtful whether soluble ASGP-R would.

#### **4.4.5. Limitations of using the ASGP-R**

Baenziger and Fiete (Baenziger & Fiete, 1980) found that a peptide with four galactose-terminating mono-antennary sugar chains can be bound and endocytosed by the ASGP-R of rat hepatocytes, whereas two such sugar chains were insufficient for internalisation; the implication being that a critical number of exposed galactose residues is required before endocytosis will take place. This would constitute a significant limitation. Epo with one or two chains featuring a single exposed galactose would presumably not be cleared.

The affinity to N-acetylgalactosamine of ASGP-R is roughly a hundred times that to galactose (Iobst & Drickamer, 1996). While this could potentially give misleading results, GalNAc $\beta$ 1 $\rightarrow$ 4GlcNAc $\beta$  oligosaccharide structures have not been detected in

recombinant glycoproteins produced in CHO cells, the most commonly used production cell line (Smith *et al*, 1990).

#### **4.5. Conclusion**

In conclusion, a mammalian expression vector has been derived which should offer stable, constitutive co-expression of the H1 and H2b subunits of the ASGP-R. The Epo-dependent AS-E2 cell line was stably transfected with the ASGP-R expression vector. H1 subunit – but not H2b subunit - expression was confirmed by Western blot. The resulting genetically engineered cell line was thus rendered capable, to a certain extent, of differentiating between intact and desialylated Epo.

## **Chapter 5. Development of a sialylation-sensitive cell-based bioassay; genetic manipulation of an Epo-dependent cell line to display cell-surface galactose-specific plant lectins**

### **Summary and Overview**

Chapter 5 describes an attempt to develop a sialylation-sensitive *in vitro* assay by stable transfection of an Epo-dependent cell line with a vector encoding a galactose-binding plant lectin. It features the following sequence of studies:

- (i) Isolation of cDNAs encoding galactose-binding plant lectins and mammalian transmembrane domains and their ligation into a mammalian expression vector (Sections 5.2.1 - 6);
- (ii) Attempt to detect expression by Western blot and fluorescence microscopy (Sections 5.3.3. and 5.3.4.);
- (iii) Comparison of the potencies of intact and desialylated Epo obtained in assays employing untransfected/empty vector-transfected and Epo-dependent cells stably transfected with a vector encoding a transmembrane-directed galactose-binding plant lectin (Section 5.3.5.);
- (iv) Discussion of the results (Section 5.4.).

### **5.1. Introduction and aims of the study**

Chapter 4 described an assay in which Epo-dependent AS-E2 cells were stably transfected with the galactose/N-acetylgalactosamine-specific asialoglycoprotein receptor. This receptor acted as a decoy receptor, binding desialylated Epo and depleting the quantities present in the surrounding medium. The objective of the project outlined in this chapter was similar, but aimed to exploit the properties of the

plant lectins described in Chapter 3 by engineering cells to display them at their surface. These proteins also have an affinity for carbohydrates but with different, and potentially more useful, binding characteristics compared with the ASGP-R. Lectins from some plant species have stronger binding affinities for galactose and binding is not as dependent upon the oligosaccharide ligand having three terminal galactose residues. The hypothesis was that an Epo-dependent cell line displaying cell-surface plant lectins would be more sensitive and better able to resolve the differences between fully sialylated and desialylated Epo samples, rather than exhibit a bias towards tri-galactose terminating sugar chains.

#### **5.1.1. Advantages of plant lectins over the ASGP-R**

Asialo-fetuin binds the ASGP-R on hepatocyte membranes with a  $K_d$  value of 18 nM (Maitra *et al*, 1990). In contrast, the extensively studied galactose-binding plant lectin, ricin B chain, bound to asialo-fetuin with higher affinity ( $K_d$  of 9 nM, (Frankel *et al*, 1996)).

Another paper reported  $K_d$  values of 0.124 nM and 0.094 nM for two different galactose-binding sites regarding the binding of ricin B chain to immobilised asialo-fetuin (Wales *et al*, 1991). While the ASGP-R and ricin B chain were unfortunately not compared directly in the same study, the indications are that the ASGP-R binds with weaker affinity to the same ligand compared with the B chain of ricin.

These affinity binding studies used completely desialylated fetuin, but an important consideration is the more rapid decline in ASGP-R-binding activity, compared with the galactose-binding plant lectins, as the number of galactose-terminated branches decreases. Studies using *Ricinus communis* agglutinin and *Erythrina indica* as plant



lectin models revealed that their binding to galactose-terminating sugars was based on a single statistical model rather than the "cluster effect" described in Chapter 1. Any increase in binding was consistent with an increase in the local concentration of galactose residues (Bhattacharyya & Brewer, 1988).

Asialotransferrin contains one bi-antennary glycans structure and one tri-antennary glycans. If just one branch is sialylated, the amount which accumulates in the liver is halved, illustrating how the action of the liver is severely affected in the absence of a minimum number of exposed galactose residues (Regoeczi *et al*, 1980). In essence, the rate of clearance was not proportional to the number of galactose residues exposed, which may have implications for the sensitivity of the assay when used to compare less desialylated Epo samples.

There are several galactose-binding lectins, including lectins from *Ricinus communis*, *Erythrina crista-galli*, *Arachis hypogaea* (peanut), *Artocarpus integrifolia* and *Vicia villosa*. *R. communis* and *E. crista-galli* would be suitable for use in an assay involved in binding galactose-exposing glycoproteins as these two lectins bind galactose with the commonly found Gal $\beta$ 1-4GlcNAc $\beta$ 1 linkage. *A. hypogaea*, *A. integrifolia* and *Vicia villosa* would have limited use in such an assay as they bind types of galactose residue (Gal $\beta$ 1-3GalNAc $\alpha$ 1-Ser/Thr or GalNAc $\alpha$ -Ser/Thr) that are rarely found in mammalian glycoproteins.

### **5.1.2. *Erythrina crista-galli* lectin**

Of all the galactose-binding plant lectins, *E. crista-galli* lectin (ECL), the lectin used in Chapter 3, appeared the most promising for use in an assay designed to target galactose-exposing glycoproteins. As well as preferentially binding saccharides terminating in Gal $\beta$ (1,4)GlcNAc, it also binds with the highest affinity. Gupta *et al*

(1993) used inhibition of haemagglutination assays and found that ECL binds such ligands with higher affinity than do *E. corallodendron* and *E. indica* lectins, and with roughly twice as great an affinity as ricin. Debray *et al* (1986) compared four *Erythrina* lectins using immobilised lectin columns and analysed elution profiles of galactose-containing oligosaccharides. *E. crista-galli* consistently had the highest affinity compared with *E. corallodendron*, *E. lysistemon* and *E. latissima*. ECL, a 241 amino acid-long divalent ion-dependent protein (Stancombe *et al*, 2003; Svensson *et al*, 2002), is derived from the Cockspur coral tree, *E. crista-galli*. It takes on a homodimeric structure, the dimer interface being made up of mostly hydrophobic interactions (Svensson *et al*, 2002), which was thought might affect its ability to be displayed as a monomer on the surface of a cell. Accordingly, the galactose-binding lectins of *R. communis* were also chosen as candidates for cell-surface display owing to their ability to exist in monomeric form (Venkatesh & Lambert, 1997).

### **5.1.3. The *Ricinus communis* lectins**

*R. communis* seeds contain two lectins: ricin (RCA<sub>60</sub>), of molecular weight 60 kDa, and *Ricinus communis* agglutinin (RCA<sub>120</sub>), of 120 kDa (Debray *et al*, 1981). RCA<sub>60</sub> is a toxin composed of a cytotoxic A chain (267 amino acids) and a galactose-binding B chain (262 amino acids) (Hegde & Podder, 1998). The B chain binds galactose residues exposed on a cell's surface, thus enabling the toxic A chain to enter the cell and inhibit protein synthesis by cleaving an adenine base from the ribosomal RNA (Hegde & Podder, 1998). RCA<sub>120</sub> is a tetramer consisting of two dimers similar to ricin, held together by non-covalent forces. It is less toxic than RCA<sub>60</sub>. The B chain folds into two globular domains, each of which binds to a single galactose (Sphyris *et*

*al*, 1995). Venkatesh and Lambert (1997) blocked the two galactose-binding sites but, having shown that there was still residual galactose-binding activity, concluded that there must be a third site. The A and B chains are linked by an intra-disulphide bridge and yet are able to remain associated even in its absence through hydrophobic interactions (Hegde & Podder, 1998). The genes for ricin and *Ricinus communis* agglutinin were first cloned in 1985 from the castor bean (*Ricinus communis*) (Halling *et al*, 1985; Roberts *et al*, 1985). Their B chains share 84% homology, with all but 41 residues being identical.

Studies have focused on the inhibition of red blood cell agglutination in an attempt to identify the differences in binding characteristics between the two *R. communis* lectins (Debray *et al*, 1981). RCA<sub>60</sub> binds oligosaccharides terminating in galactose residues with two-to-fourfold greater affinity, but its drawback in terms of an assay is that it cannot differentiate as effectively between non-sialylated and sialylated forms of sugar, having slightly greater affinity for sialic acid-terminating oligosaccharide structures than does RCA<sub>120</sub> (Debray *et al*, 1981). It was therefore decided to try both. Low levels of sialyl- $\alpha$ (2 $\rightarrow$ 6)-lactose binding by RCA<sub>120</sub> were detected, but not of sialyl- $\alpha$ (2 $\rightarrow$ 3)-lactose (Debray *et al*, 1981). This was attributed to the former being more accessible to the lectin due to the sialyl group's greater degree of rotational freedom. Any binding of sialylated sugars would adversely interfere with the assay, the principle aim of which is to selectively sequester only galactose-terminating glycoproteins. This is not a major problem, though, as Chinese Hamster Ovary (CHO) cells, the cells used to produce biotherapeutic glycoproteins, do not possess the alpha-2,6-sialyltransferase (Ma *et al*, 1997).

#### **5.1.4. Designing chimeric decoy receptors**

The aim of this part of the project was to produce a chimeric galactose-binding decoy receptor consisting of an N-terminal signal peptide, an extracellular plant lectin domain, a flexible linker and a transmembrane domain. Fig. 5.1.1. is a schematic representation of the chimeric decoy receptor.

The ricin B chain has previously been expressed as a fusion protein on the surface of fd phage (Swimmer *et al*, 1992) so as to be able to screen a library of mutants of the lectin for altered binding activities. The wild-type form was still able to bind sugars as a monomer and could tolerate being part of a fusion protein and having extra domains attached C-terminally. It did, however, differ in that it was not displayed on the surface membrane of a cell.

The plant lectins would be expected to fold correctly after being extruded through the cell membrane, as the endogenous forms have plant-specific signal peptides that direct their extrusion through the plant cell membrane (Hegde & Podder, 1998; Stancombe *et al*, 2003). The ricin B chain has also been expressed in *E. coli* fused with a heterologous signal peptide directing its secretion into the periplasmic space (Hussain *et al*, 1989). In the case of the project described in this chapter, the lectin constructs were instead engineered in such a way as to contain the mammalian Epo-R signal sequence.

The ricin B chain expressed as a fusion protein on the surface of fd phage contained a [Ser-Gly<sub>3</sub>]<sub>4</sub> flexible amino acid linker whose purpose was to facilitate presentation of the recombinant lectin as a separately folded domain (Swimmer *et al*, 1992). The chimeric receptors discussed in this chapter contained a similar [Gly<sub>4</sub>Ser]<sub>4</sub> flexible linker.

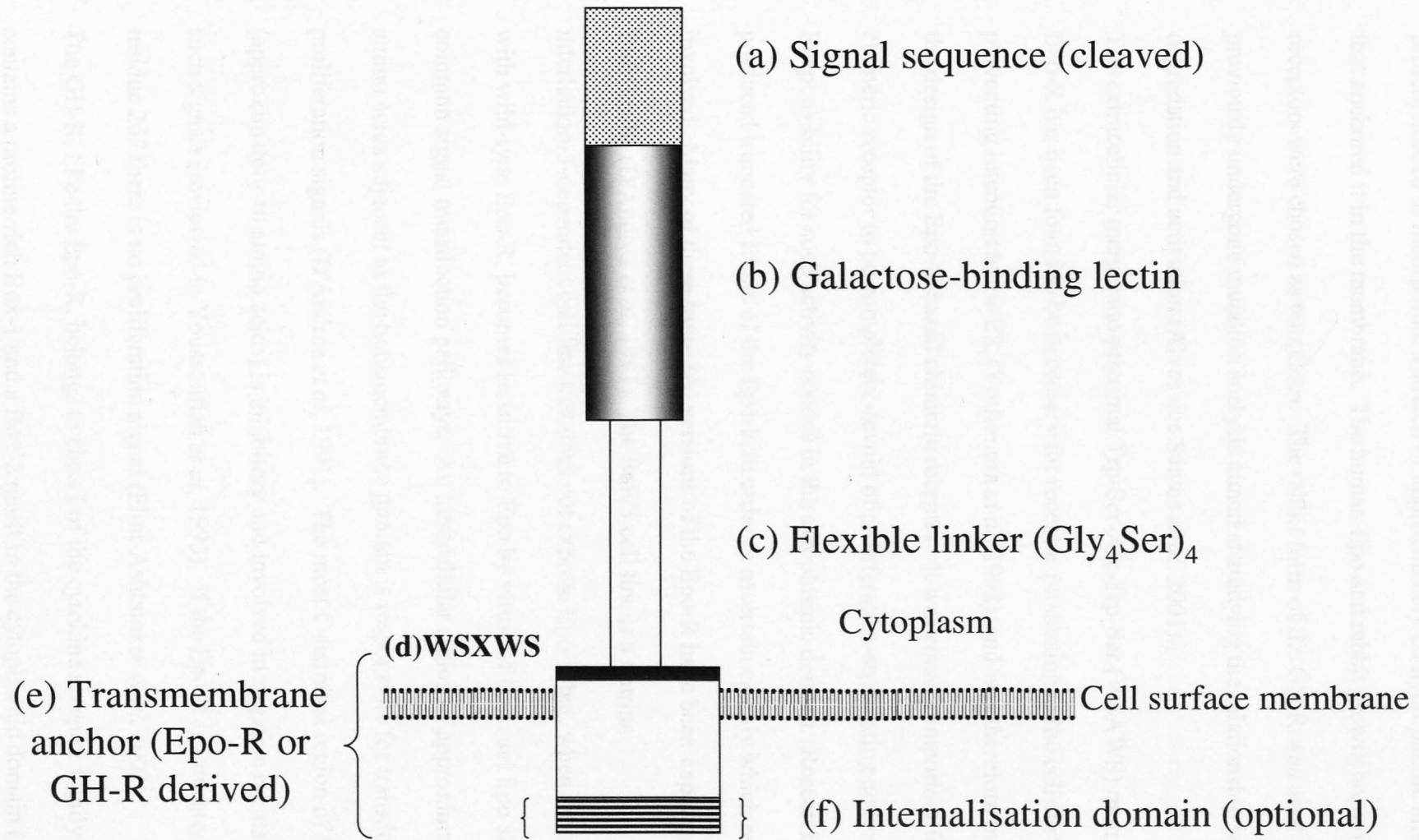
Fig. 5.1.1. (Facing page) Schematic representation of the design of a surface-expressed decoy receptor incorporating a galactose-binding plant lectin domain.

The signal peptide (a) is responsible for directing the galactose-binding lectin (b) to and through the cell surface membrane.

The flexible linker (c) ensures the lectin is presented as a separately folded domain.

The Epo-R's WSAWS motif (d) - YEGFS in the case of the GH-R - is included in view of its importance in terms of processing the Epo-R to the cell surface and preventing retention in the endoplasmic reticulum.

The transmembrane domain (e) derived from either the Epo-R or mutant GH-R (the latter containing an internalisation motif (f) but lacking any signal-transduction capability) anchors the fusion protein in the cell surface membrane.



For the galactose-binding plant lectins to be displayed on the cell's surface, the fusion protein needed to incorporate a stretch of approximately 20 hydrophobic amino acids that anchored it in the membrane. The human Epo and rabbit growth hormone (GH) receptors were chosen as templates. The rabbit form of the GH-R was used as it had previously undergone mutation analysis aimed at studying the relationship between degradation and activation (Alves dos Santos *et al*, 2001).

The extracellular membrane-proximal Trp-Ser-Ala-Trp-Ser (WSAWS) motif of the Epo-R has been found to be necessary for receptor processing to the cell surface and preventing retention in the ER (Yoshimura *et al*, 1992), and was therefore included in the design of the Epo-R-based chimeric receptor. It was equally important for any chimeric receptor to be completely devoid of proliferation-stimulating activity.

Responsibility for such activity resides in the cytoplasmic domain. Researchers have produced truncated forms of the Epo-R in order to ascertain exactly which regions are involved. Many of these truncated versions of the Epo-R have been expressed in BaF/3 cells (D'Andrea *et al*, 1991). The BaF/3 cell line is a murine interleukin-3-dependent cell line that does not express Epo-R but, when transfected with wild-type Epo-R, becomes sensitive to Epo by virtue of IL-3 and Epo sharing common signal transduction pathways. An intracellular region of approximately 100 amino acids adjacent to the transmembrane domain is responsible for transduction of proliferation signals (D'Andrea *et al*, 1991). The most C-terminal region of Epo-R (approximately 40 amino acids) is inhibitory and involved in negative regulation of such signals (reviewed in Youssoufian *et al*, 1993). If the Epo-R is truncated at residue 267 there is no proliferation signal (Flint-Ashtamker *et al*, 2002).

The GH-R, like the Epo-R, belongs to class I of the cytokine receptor family and so contains a proline-rich Box-1 and a Box-2 motif in the cytoplasmic domain (Strous &

van Kerkhof, 2002). Rabbit GH-R contains a total of 620 amino acid residues, of which 246 are extracellular, 24 constitute the transmembrane domain, and 350 are intracellular (Leung *et al*, 1987). When rabbit GH-R had four of its box-1 domain prolines changed to alanines, it no longer transmitted proliferation signals but was still able to undergo degradation (Alves dos Santos *et al*, 2001). These are potentially useful properties for a decoy receptor whose aim it is to remove certain populations of Epo molecules from the medium without inducing proliferation.

The work outlined in this chapter involved creating fusion constructs made up of regions from plant and animal proteins and, while it is true that exchanging cytokine receptor regions has been carried out (cf. Epo-R/epidermal growth factor receptor (EGF-R) (Pacifici & Thomason, 1994) and GH-R/granulocyte colony-stimulating factor receptor [G-CSF-R] chimeras (Ishizaka-Ikeda *et al*, 1993)) and can be tolerated in terms of the fact that the chimeric receptors can still transduce proliferation signals, it is not known if a plant lectin can replace a cytokine receptor domain.

## **5.2. Experimental Procedures**

### **5.2.1. RNA isolation and first-strand cDNA synthesis**

Total RNA was isolated from *E. crista-galli* and *R. communis* seeds using a plant RNeasy Plant mini-kit (Qiagen), whilst messenger RNA was extracted from AS-E2 cells using the QuickPrep Micro mRNA purification kit from Amersham (as described in Section 2.1.1.). First-strand cDNA synthesis was performed according to Section 2.1.2.



### **5.2.2. PCR-amplification of galactose-binding plant lectins**

*The plant lectins were PCR-amplified (Section 2.1.3.) without their signal peptides from the appropriate seed first-strand cDNA using the following primers:*

Name	Function	Primer sequence
ECL-5'	Forward primer for ECL gene	5'-gaaaccatatcgtttagcttcagcg-3'
ECL-3'	Reverse primer for ECL gene	5'-atcgttgtttctggcaacgaggc-3'
RCA-5'	Forward primer suitable for amplification of both RCA <sub>60</sub> and RCA <sub>120</sub> genes	5'-gctgatgtttgtatggatcctgag-3'
RCA-3'	Reverse primer suitable for both RCA <sub>60</sub> and RCA <sub>120</sub> genes	5'-aaataatggaacatatttg-3'

PCR products were run on a 1% agarose gel to verify that they were of the correct size (see Fig. 5.2.1. for amplification of the ECL gene and Fig. 5.2.2. for amplification of what is presumably a mixture of the two RCA genes). The PCR product was ligated into pGEM-T (Promega) by TA cloning, and then transformed into JM109 bacteria (Sections 2.1.4. and 2.1.5. respectively). Identification of the ECL, RCA<sub>60</sub> and RCA<sub>120</sub> genes was achieved by sequencing (Section 2.1.10.) and comparison with GenEMBL Accession numbers AY158072 (Stancombe *et al*, 2003), E01356 and E01357 respectively. Sequences of the clones were verified as correct.

#### **5.2.2.1. Site-directed mutagenesis (SDM)**

Cysteine<sup>4</sup> of RCA<sub>60</sub> and RCA<sub>120</sub> was changed to a serine by site-directed mutagenesis (following the protocol in Section 2.1.14.), as carried out by Swimmer *et al* (1992).

The RCA<sub>60</sub> and RCA<sub>120</sub> B chains have four intrasubunit disulfide bridges and a

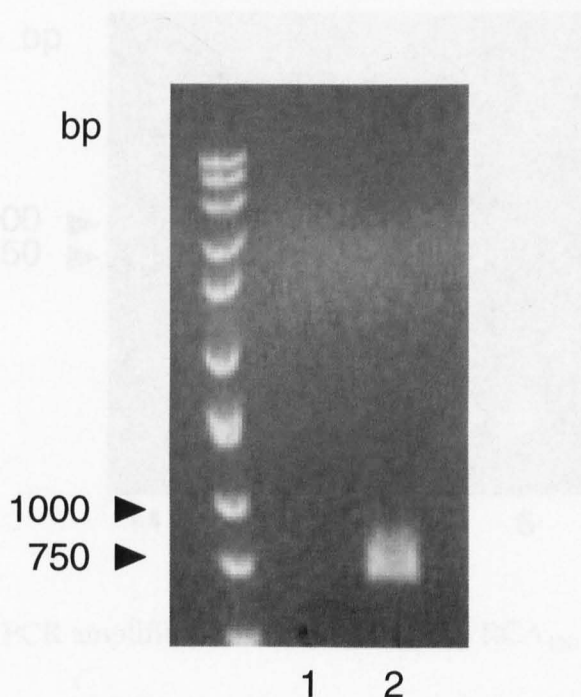


Fig. 5.2.1. PCR amplification of *Erythrina crista-galli* lectin using the listed primers and *Erythrina crista-galli* first-strand cDNA as a template. Ethidium Bromide-stained 1% agarose gel loaded as follows:

- Lane 1; primers ECL-5' and ECL-3' (2  $\mu$ M final concentration).
- Lane 2; primers ECL-5' and ECL-3' (20  $\mu$ M).

Expected product size= ~710 bp.

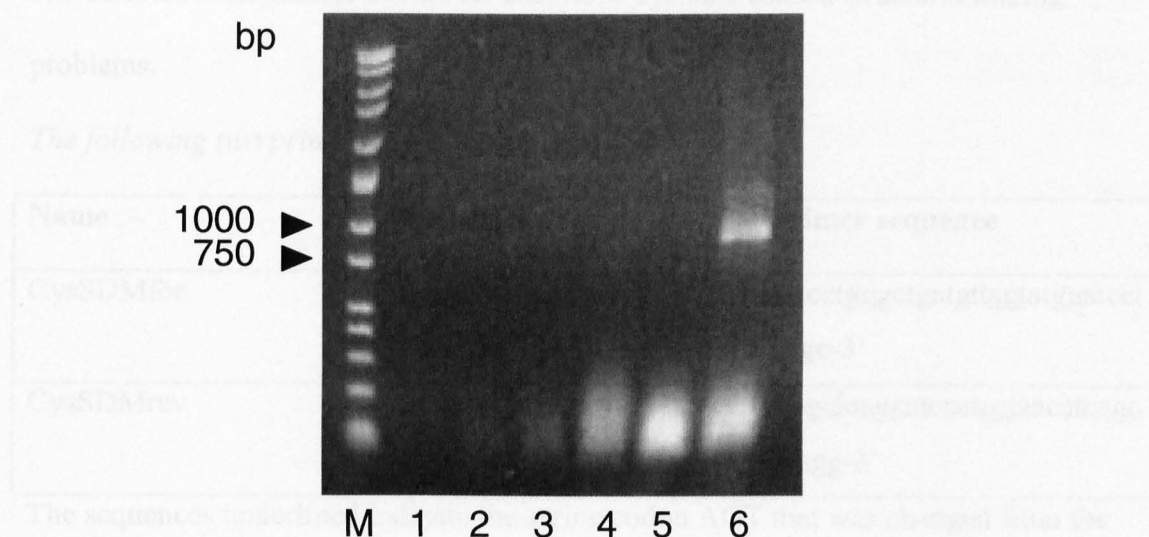


Fig. 5.2.2. PCR amplification of the RCA<sub>60</sub> and RCA<sub>120</sub> B chains using RCA-5' and RCA-3' primers (complementary to both genes) and *Ricinus communis* first-strand cDNA as a template. Ethidium Bromide-stained 1% agarose gel loaded as follows:

-Lane 1; 2  $\mu$ M (primer final concentration), 1 mM MgCl<sub>2</sub>.

-Lane 2; 2  $\mu$ M, 1.5 mM MgCl<sub>2</sub>.

-Lane 3; 2  $\mu$ M, 1 mM MgCl<sub>2</sub>.

-Lane 4; 20  $\mu$ M, 2 mM MgCl<sub>2</sub>.

-Lane 5; 20  $\mu$ M, 1.5 mM MgCl<sub>2</sub>.

-Lane 6; 20  $\mu$ M, 2 mM MgCl<sub>2</sub>.

Expected product size= ~780 bp.

remaining half-cysteine (cys<sup>4</sup>) which forms an intersubunit disulfide bridge with Cys<sup>299</sup> of the A chain. It was decided to change this residue to a serine by site-directed mutagenesis in case an unbonded cysteine caused structural folding problems.

*The following two primers were used for the SDM:*

Name	Function	Primer sequence
CysSDMfor	Forward primer for changing cys <sup>4</sup> to ser	5'-cctgggctgatgtagtatggatcctgagc-3'
CysSDMrev	Reverse primer for changing cys <sup>4</sup> to ser	5'-gctcaggatccataactaacatcagccagg-3'

The sequences underlined indicate the serine codon AGT that was changed from the cysteine codon TGT.

### **5.2.3. Introduction of a 5' Epo receptor signal peptide by PCR**

The plant lectin-encoding vectors described in Section 5.2.2. were then used as template DNA for addition by PCR of the Epo-R (GenEMBL accession number M60459) signal peptide, initial attempts to do this from plant RNA having been unsuccessful.

The following primers were used (RCA primers being suitable for both  $RCA_{60}$  and  $RCA_{120}$  genes, owing to their high percentage homology):

Name	Function	Primer sequence	Restriction site
Sig pep-ECL-5'	Forward primer for introducing Epo-R signal peptide at 5' end of ECL gene	5'-gacgatgcccgcgggctg tatcatggaccacctcggggcgctc cctctggccccaggctgggtccct ttgtctctgctcgctggggccgc ctgggtggaaaccatategttta gcttcagcg-3'	-NotI underlined -ECL complementary region in bold
ECL/ <i>Pst</i> I-3'*	Reverse primer for introducing <i>Pst</i> I restriction site 3' of the ECL gene	5'-gacgatctgcagatcgttgttt ctggcaacgaggc-3'	- <i>Pst</i> I underlined
Sig pep-RCA-5'	Forward primer for introducing Epo-R signal peptide at 5' end of $RCA_{60}$ or $RCA_{120}$ gene	5'-gacgatgcccgcgggctg tatcatggaccacctcggggcgctc cctctggccccaggctgggtccct ttgtctctgctcgctggggccgc ctgggtgatgtttgtatggatcc tgag-3'	-NotI underlined -RCA complementary region in bold
RCA/ <i>Pst</i> I-3'*	Reverse primer for introducing <i>Pst</i> I restriction site 3' of the RCA genes	5'-gacgatctgcagaaataatgg taaccatatttg-3'	- <i>Pst</i> I underlined

\*The *Pst*I restriction sites were needed for assembly of the lectin construct later in the procedure.

PCR products were run on a 1% agarose gel to check that they were of the correct size (Fig. 5.2.3.), re-subcloned into pGEM-T, transformed into bacteria, and analysed by NotI restriction digestion. Only clones that contained genes inserted in such a way as to ensure that the 5'-end was next to the pGEM-T T7 promoter (as identified by the presence of a NotI fragment) were selected. This step was necessary to permit

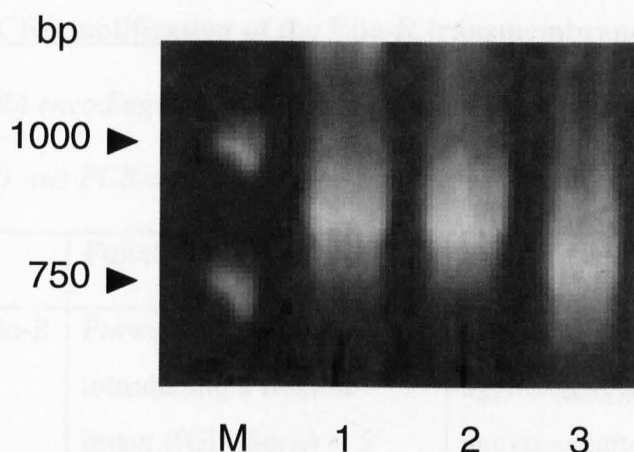


Fig. 5.2.3. PCR amplification of plant lectins (ECL, RCA<sub>60</sub> and RCA<sub>120</sub> B chains) with incorporation of the Epo-R signal peptide, using the listed primers and plant lectin cDNA-containing pGEM-T vectors described in Section 5.2.2. as a template. Ethidium Bromide-stained 1% agarose gel loaded as follows (all primers used at 20  $\mu$ M final concentration):

-Lane 1; primers Sig pep-RCA-5' and RCA/*Pst*I-3' using pGEM-T[RCA<sub>60</sub>] as a template.

-Lane 2; primers Sig pep-RCA-5' and RCA/*Pst*I-3' using pGEM-T[RCA<sub>120</sub>] as a template.

-Lane 3; primers Sig pep-ECL-5' and ECL/*Pst*I-3' using pGEM-T[ECL] as a template.

subsequent assembly of the gene fusion construct. Such clones were further verified by sequencing.

#### **5.2.4. PCR-amplification of the Epo-R transmembrane domain**

*The cDNA encoding the transmembrane domain of the Epo-R (amino acid residues 200-267) was PCR-amplified using the following primers:*

<b>Name</b>	<b>Function</b>	<b>Primer sequence</b>	<b>Restriction site</b>
Flink/Epo-R TM-5'	Forward primer for introducing a flexible linker ([Gly <sub>4</sub> Ser] <sub>4</sub> ) at 5' end of the Epo-R transmembrane domain	5'-gacgatctgcagggaggtg aggttcaggaggtggaggttcag gaggtggaggttcaggaggtgga gggtcagctgagccgagcttcgg cggttc-3'	-PstI underlined -Epo-R complementary region in bold
Epo-R TM-3'	Reverse primer for introducing stop codon into Epo-R in order to produce a truncated form of the receptor (see text for details)	5'-gacgatgagctccagcaca ctggtactcgtctctctgggctgc cgatgc-3'	-SacI underlined -BstXI in italics -Stop codon in bold

The PCR products (see Fig 5.2.4.) were cloned into the pGEM-T vector (Promega) and transformed into JM109 bacteria. The Epo-R transmembrane sequence was compared with GenEMBL Accession No. M60459 and verified as correct.

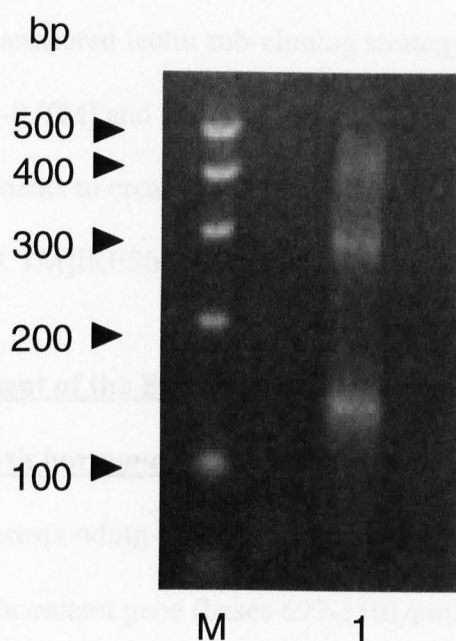


Fig. 5.2.4. PCR amplification of the Epo-R transmembrane domain incorporating a 5' flexible linker using the listed primers and AS-E2 first-strand cDNA as a template. Ethidium Bromide-stained 1% agarose gel loaded as follows:

-Lane 1; primers Flink/Epo-R TM-5' and Epo-R TM-3' (20  $\mu$ M final concentration). Amplified region consists of the EpoR transmembrane domain (~200 bp) plus a 70 bp artificial linker. Bands seen at ~150 bp and ~400 bp are presumed to be non-specific.



### **5.2.5. Assembly of the plant lectin/Epo-R chimeric construct and ligation into pIRESblast**

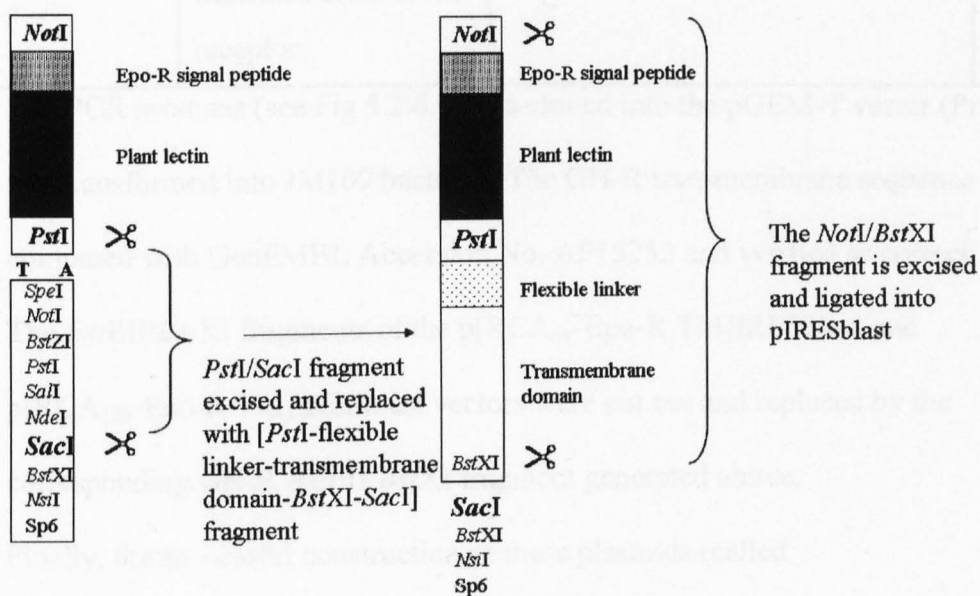
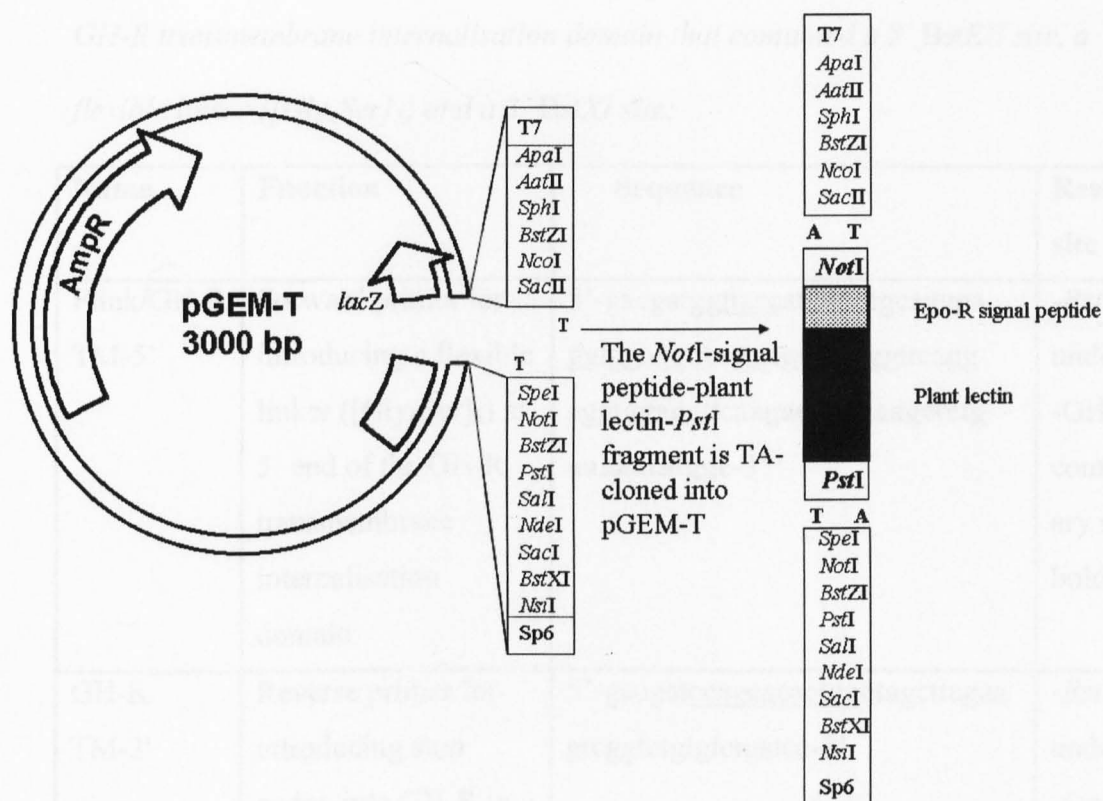
The next step was to ligate the plant lectin genes to the Epo-R transmembrane encoding region. See Fig. 5.2.5. for a schematic representation of the chimeric transmembrane-anchored lectin sub-cloning strategy. The final step involved ligating the [RCA<sub>60</sub>-Epo-R TM] and [RCA<sub>120</sub>-Epo-R TM] constructs into pIRESblast as *NotI*/*Bst*XI fragments to create vectors p[RCA<sub>60</sub>-Epo-R TM]IRESblast and p[RCA<sub>120</sub>-Epo-R TM]IRESblast respectively.

### **5.2.6. Replacement of the Epo-R transmembrane domain-encoding region with that of the growth hormone receptor transmembrane and degradation domains**

GH-R cDNA corresponding to the transmembrane and degradation domains of the GH-R 4Pro →Ala mutant gene (bases 697-1101/amino acids 233-367) were PCR-amplified with appropriate restriction sites at both ends using the rGHRBOX1outknock plasmid (generously donated from Dr. G. Strous, University Medical Centre, Utrecht, Netherlands) as a template.

Fig. 5.2.5. (Facing page) Schematic representation of the sub-cloning strategy for constructing a mammalian expression vector encoding a transmembrane domain-anchored plant lectin. An in-frame fusion is used to create an open reading frame encoding a protein that extends from the signal sequence of the Epo-R gene (amino acids 1-24) into the ricin or *Ricinus communis* agglutinin B chain, followed by the flexible linker and, lastly, the region of the EpoR gene that spans the WSXWS motif, the transmembrane domain and the cytoplasmic domain as far as residue 268.

The cassettes encoding the fusion protein constructs were then cloned into pIRESblast as NotI/BstXI fragments.



*A unique BstEII restriction site was identified just upstream of the Epo-R flexible linker and the transmembrane domain and so primers were designed to amplify the GH-R transmembrane internalisation domain that contained a 5' BstEII site, a flexible linker ([Gly<sub>4</sub>Ser]<sub>3</sub>) and a 3' BstXI site:*

Name	Function	Sequence	Restriction site
Flink/GH-R TM-5'	Forward primer for introducing a flexible linker ([Gly <sub>4</sub> Ser] <sub>3</sub> ) at 5' end of the GH-R transmembrane internalisation domain	5'-gacgat <u>ggttaccattatttctgcaggga</u> ggtggagggttcaggaggtggaggttcagg aggtggagggttcaagacagcgaagctctg <b>aaaaatatggc</b> -3'	-BstEII underlined -GH-R complement- ary region in bold
GH-R TM-3'	Reverse primer for introducing stop codon into GH-R in order to produce a truncated form of the receptor	5'-gacgatcagcacactggctagcttagaa gtcggctctgtgtctgatcc-3'	-BstXI underlined -Stop codon in bold

The PCR products (see Fig 5.2.6.) were cloned into the pGEM-T vector (Promega)

and transformed into JM109 bacteria. The GH-R transmembrane sequence was

compared with GenEMBL Accession No. AF15252 and verified as correct.

The BstEII/BstXI fragments of the p[RCA<sub>60</sub>-Epo-R TM]IRESblast and p[RCA<sub>120</sub>-Epo-R TM]IRESblast vectors were cut out and replaced by the

corresponding GH-R BstEII/BstXI fragment generated above.

Finally, the successful construction of these plasmids (called

p[RCA<sub>60</sub>-GH-R TM]IRESblast and p[RCA<sub>120</sub>-GH-R TM]IRESblast respectively) was

confirmed by nucleotide sequencing. Plasmids were purified using the Endofree

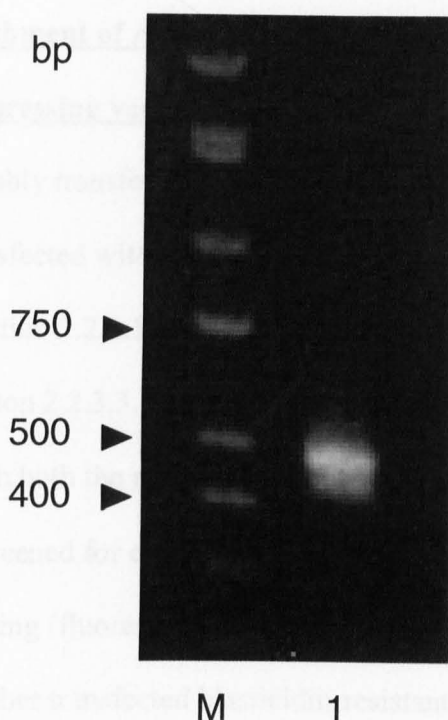


Fig. 5.2.6. PCR amplification of the Growth Hormone Receptor mutant (4Pro→Ala) transmembrane/internalisation domain with 5' *Bst*EII restriction end and flexible linker and 3' *Bst*XI restriction end using the listed primers and rGHRBOX1outknock plasmid as a template. Ethidium Bromide-stained 1% agarose gel loaded as follows:

Lane 1; Flink/GH-R TM-5' and GH-R TM-3' primers (20  $\mu$ M final concentration).

Amplified region consists of the GH-R transmembrane and internalisation domain (~400 bp), plus a 45 bp artificial linker.

Maxi Prep Kit (Qiagen) endotoxin-free purification kit according to its standard protocol.

#### **5.2.7. Establishment of AS-E2 cells stably transfected with plant lectin/TM-expressing vector constructs**

To generate stably transfected cell lines expressing plant lectin chimeric receptors, cells were transfected with endotoxin-free maxiprep DNA following the protocol outlined in Section 2.2.3.1. Resistant colonies were obtained following blasticidin selection (Section 2.2.3.3.). After being subcultured for 10 passages, AS-E2 cells transfected with both the plant lectin chimeric receptors and the empty pIRESblast vector were screened for expression of immunoactive receptor (Western blotting) and functional binding (fluorescence microscopy). PCR-amplification was used to determine whether transfected blasticidin-resistant cell lines contained recombinant DNA (Section 2.2.3.4.).

#### **5.2.8. Bioassays**

Bioassays were performed (as in Section 2.2.4.) using the AS-E2 cells stably transfected with the p[RCA<sub>60</sub>-Epo-R TM]IRESblast, p[RCA<sub>120</sub>-Epo-R TM]IRESblast, p[RCA<sub>60</sub>-GH-R TM]IRESblast and p[RCA<sub>120</sub>-GH-R TM]IRESblast. Untransfected AS-E2 cells or AS-E2 cells stably transfected with empty vector were used as the control.

#### **5.2.9. Plate plan**

The plate plan was of the same type as that used in Chapter 4 (see Fig. 4.2.3.).

### **5.3. Results**

#### **5.3.1. AS-E2 cells engineered to display ECL at the cell surface (ECL-TM) were not viable**

A total of three attempts at stable transfection of AS-E2 cells with a vector expressing a transmembrane-anchored *E. crista-galli* lectin (ECL-TM) proved unsuccessful, with all cells dying by day 28. Since, as stated in Section 5.1.2., *E. corallodendron* forms dimers with a hydrophobic dimer interface (Elgavish & Shaanan, 2001), it had been thought that ECL might not work, possibly because of a potentially unstable hydrophobic region. Although toxicity is another possible reason, treating AS-E2 cells with ECL freely present in the medium had no deleterious effect (see Fig. 3.3.8. in Chapter 3).

#### **5.3.2. Survival of AS-E2 cells stably transfected with RCA<sub>60</sub>-TM and RCA<sub>120</sub>-TM and retention of Epo-dependency**

AS-E2 cells genetically engineered to display RCA<sub>60</sub> and RCA<sub>120</sub> survived and retained their Epo-dependency. There had been a concern that Epo-dependency might have been abolished as many plant lectins have been reported to have a mitogenic effect on cells (Ashraf & Khan, 2003).

#### **5.3.3. Screening of stably transfected cell lines**

Expression of the chimeric receptors was verified by Western blot using an antibody specific for RCA<sub>60</sub> and RCA<sub>120</sub> from Vector Laboratories. Bands were not detected in p[RCA<sub>60</sub>-Epo-R TM]IRESblast- and p[RCA<sub>120</sub>-Epo-R TM]IRESblast-transfected

Fig. 5.3.1. (Facing page) SDS-PAGE/Western blot using an antibody specific for both RCA<sub>60</sub> and RCA<sub>120</sub> (Vector Laboratories) to investigate whether AS-E2 cells stably transfected with the p[RCA<sub>60/120</sub>-Epo-R TM]IRESblast vectors were successfully expressing RCA<sub>60</sub> or RCA<sub>120</sub> B chain. Triton X-100 lysates were made, run on SDS-PAGE, transferred to membrane and Western blotted with an antibody specific for RCA<sub>60</sub> and RCA<sub>120</sub> (Vector Laboratories) (following protocol in Sections 2.3.1-5.).

-Lane 1; lysate from AS-E2 cells stably transfected with the empty pIRESblast vector.

-Lane 2; lysate from AS-E2 cells stably transfected with p[RCA<sub>60</sub>-Epo-R TM]IRESblast vector encoding a membrane-anchored RCA<sub>60</sub> chimeric protein (expected molecular weight ~40 kDa).

-Lane 3; lysate from AS-E2 cells stably transfected with p[RCA<sub>120</sub>-Epo-R TM]IRESblast vector encoding a membrane-anchored RCA<sub>120</sub> chimeric protein (expected molecular weight ~40kDa).

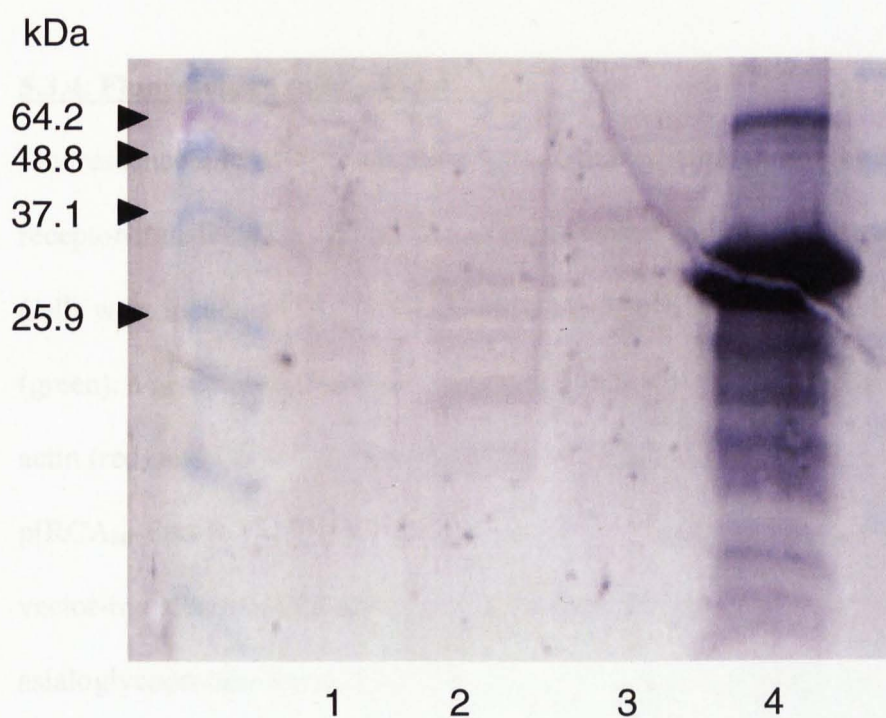
-Lane 4; Ricin B chain isolated from highly purified RCA<sub>60</sub>, molecular weight 32 kDa (Vector Laboratories).



AS-23 cells (Fig. 5.3.1). A lane was loaded with pGFP10 cells treated with highly purified RCA as a negative control.

A polyclonal anti-Tyr-1 antibody that detects the phosphorylated tyrosine residues 21-214 was used to detect the phosphorylated tyrosine residues.

potentially phosphorylated tyrosine residues of the pGFP10 cells treated with RCA as a negative control.



AS-E2 cells (Fig. 5.3.1.). A lane was loaded with ricin B chain isolated from highly purified RCA<sub>60</sub> (Vector Laboratories) as a positive control.

A polyclonal anti-Epo-R antibody from Santa Cruz Biotechnology raised against amino acids 21-214 mapping near the N-terminus of the Epo-R, and therefore potentially recognising only 14 amino acids of the RCA/Epo-R fusion proteins, also proved negative (results not shown).

#### **5.3.4. Fluorescence microscopy**

Fluorescence microscopy was used to ascertain whether or not the chimeric receptor-transfected AS-E2 cells had an increased ability to bind galactose residues. Cells were incubated in Alexa Fluor 488-conjugated asialo- $\alpha$ -1-acid glycoprotein (green), a glycoprotein containing exposed galactose residues, as well as stained for actin (red) and DNA (blue) as described in Section 2.3.13.

p[RCA<sub>60</sub>-Epo-R TM]IRESblast did not stain more strongly than empty vector-transfected cells, indicating that there was no recombinant asialoglycoprotein-binding activity (Fig. 5.3.2a and b respectively). This was also the case for p[RCA<sub>120</sub>-Epo-R TM]IRESblast and p[RCA<sub>120</sub>-GH-R TM]IRESblast-transfected cells (results not shown).

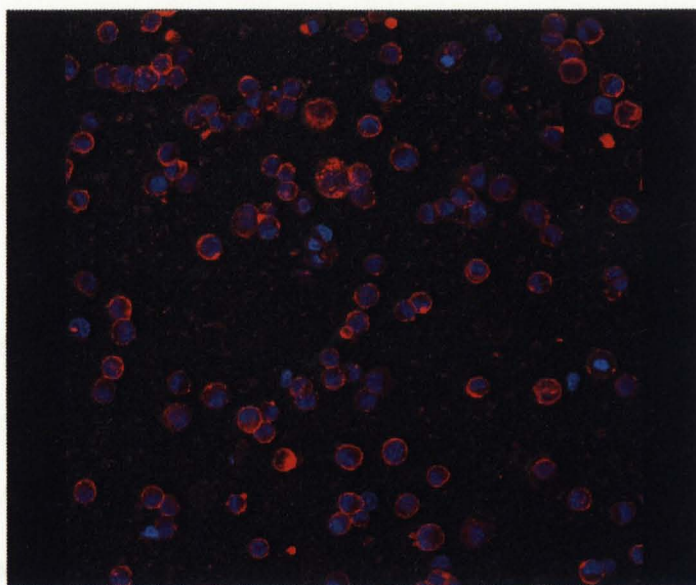
Fig. 5.3.2. (Facing page) Investigating whether expression of a functional galactose-binding chimeric protein had been achieved in lectin display vector-transfected AS-E2 cells: binding of fluorophore-labelled asialo- $\alpha$ -1-acid glycoprotein. Fluorescence images of cells stained with Phalloidin-574 (red), Hoechst (blue) and Alexa-Fluor conjugated-asialo- $\alpha$ -1-acid glycoprotein (green). See Section 2.3.13. for explanation and protocol.

(a) AS-E2 cells stably transfected with pIRESblast empty vector.

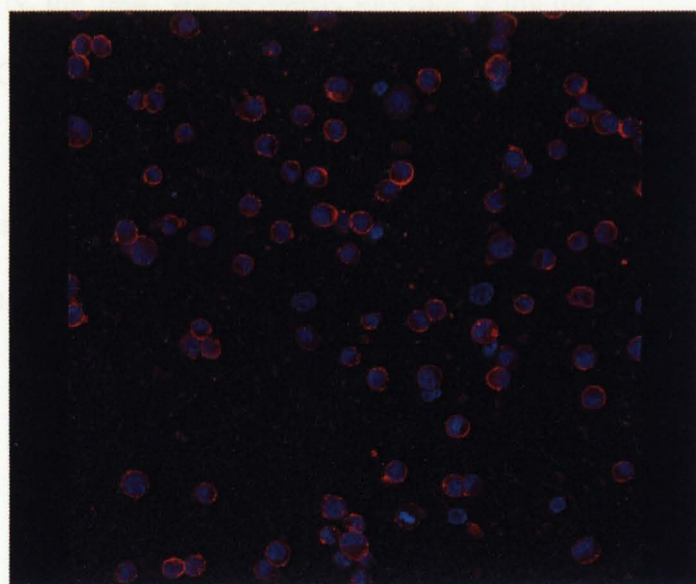
(b) AS-E2 cells stably transfected with pIRESblast[RCA<sub>60</sub>-Epo-R TM].

Cells are approximately 20  $\mu$ M in diameter.

(a)



(b)



### **5.3.5. Bioassays**

Intact Epo and the most desialylated Epo sample from Section 2.3.10. (Epo samples  $t=0$  min and  $t=1800$  min respectively) were used in assays using the various cell lines stably transfected with plant lectins. Fig. 5.3.3. shows the results comparing proliferative assays using (a) untransfected, (b), p[RCA<sub>60</sub>-Epo-R TM]IRESblast-transfected and (c) p[RCA<sub>120</sub>-Epo-R TM]IRESblast-transfected AS-E2 cells. The curves of samples  $t=0$  min and  $t=1800$  min are the same in each of the three graphs, indicating that neither of the RCA-Epo-R TM-transfected cell lines is, in relation to the untransfected AS-E2 cell line, able to distinguish between intact Epo and desialylated Epo.

If the above failure to observe an effect was due to there not being sufficient numbers of galactose-binding sites on the surface of each cell, the next logical step to take would be to see if the incorporation of an internalisation domain improved results. Accordingly, the assay was repeated, this time using the p[RCA<sub>60</sub>-GH-R TM]IRESblast- and p[RCA<sub>120</sub>-GH-R TM]IRESblast-transfected cell lines (Fig. 5.3.4.). No differentiation was made between samples  $t=0$  min and  $t=1800$  min, however, consistent with there having been no chimeric receptor expression in the first instance.

Fig. 5.3.3. (Facing page) Proliferative response of AS-E2 cells stably transfected with the empty pIRESblast, pIRESblast[RCA<sub>60</sub>-Epo-R TM] and pIRESblast[RCA<sub>120</sub>-Epo-R TM] vectors to intact and extensively desialylated Epo (samples t=0 min and t=1800 min respectively from Chapter 3).

(a) AS-E2 cells transfected with the empty pIRESblast vector.

(b) AS-E2 cells transfected with the RCA<sub>60</sub>-encoding vector (pIRESblast[RCA<sub>60</sub>-Epo-R TM]).

(c) AS-E2 cells transfected with the RCA<sub>120</sub>-encoding vector (pIRESblast[RCA<sub>120</sub>-Epo-R TM]).

Sample t=0 min (■) and sample t=1800 min (●). Data represent mean +/- s.e.m. of triplicated samples.

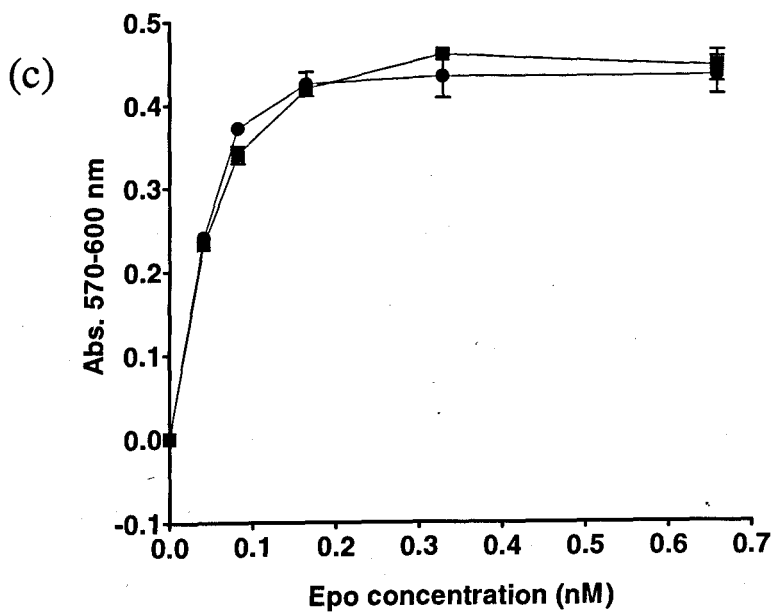
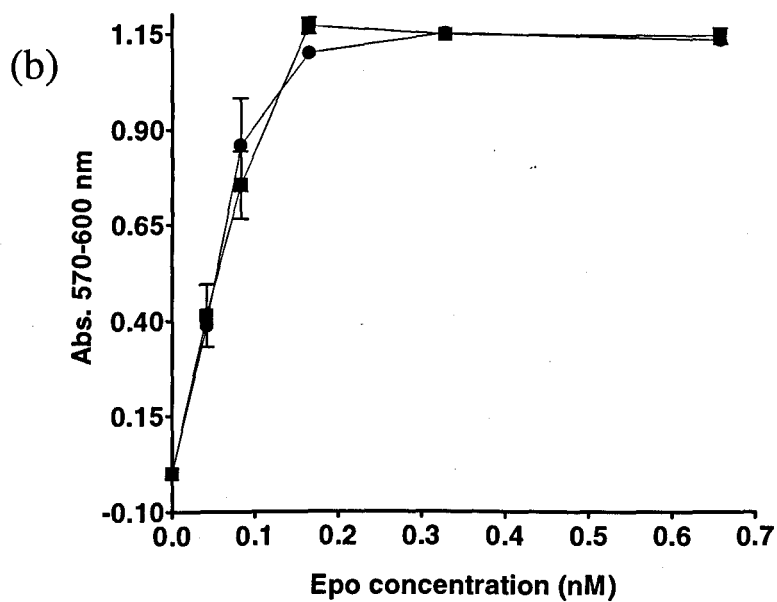
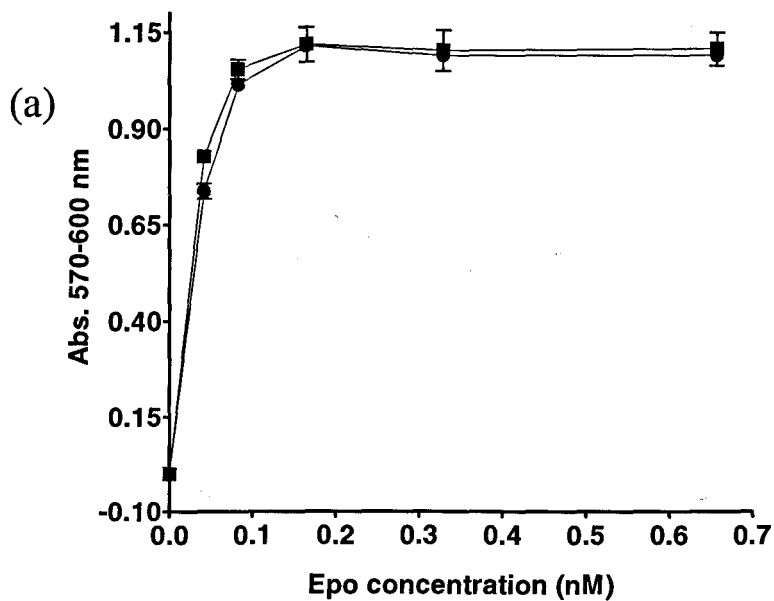


Fig. 5.3.4. (Facing page) Proliferative response of untransfected AS-E2 cells and AS-E2 cells stably transfected with the RCA<sub>60</sub> or RCA<sub>120</sub> B chain gene fused to the transmembrane domain of the mutant growth hormone receptor (activation disabled but internalisation competent) to intact and extensively desialylated Epo (samples t=0 min and t=1800 min respectively from Chapter 3).

(a) Untransfected AS-E2 cells.

(b) AS-E2 cells transfected with the RCA<sub>60</sub>-encoding vector

(pIRESblast[RCA<sub>60</sub>-GH-R TM]).

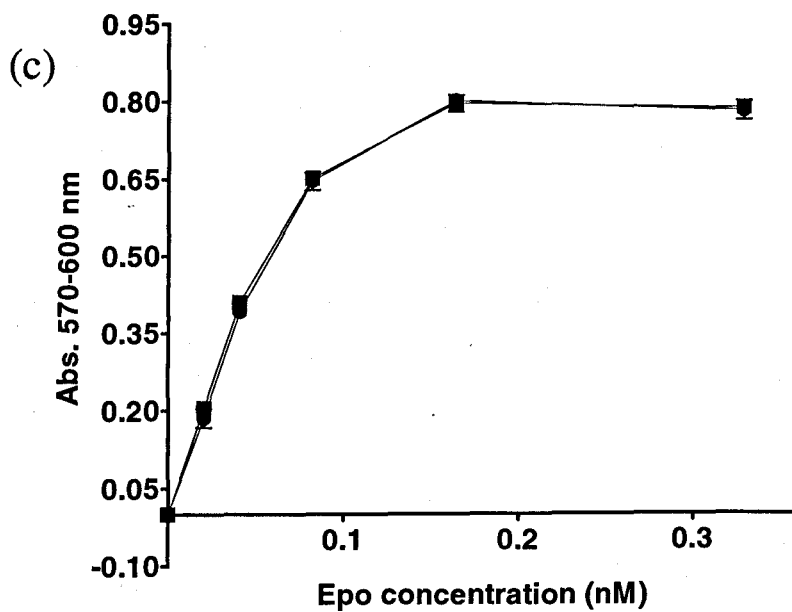
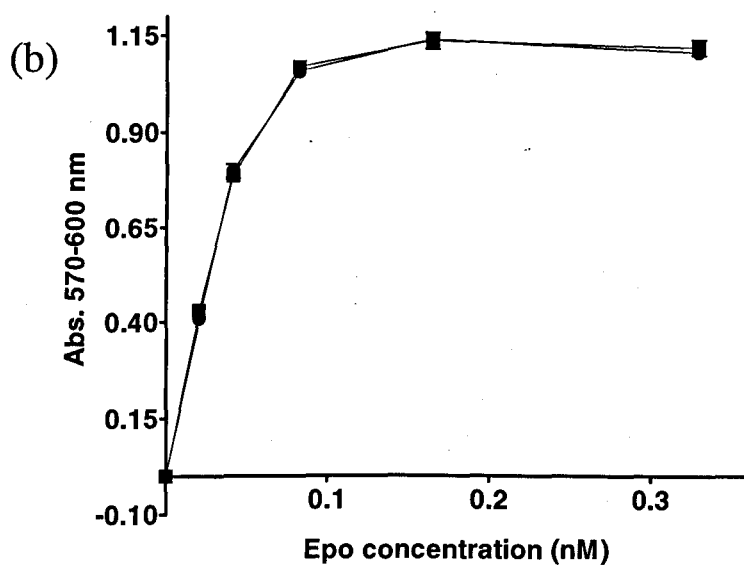
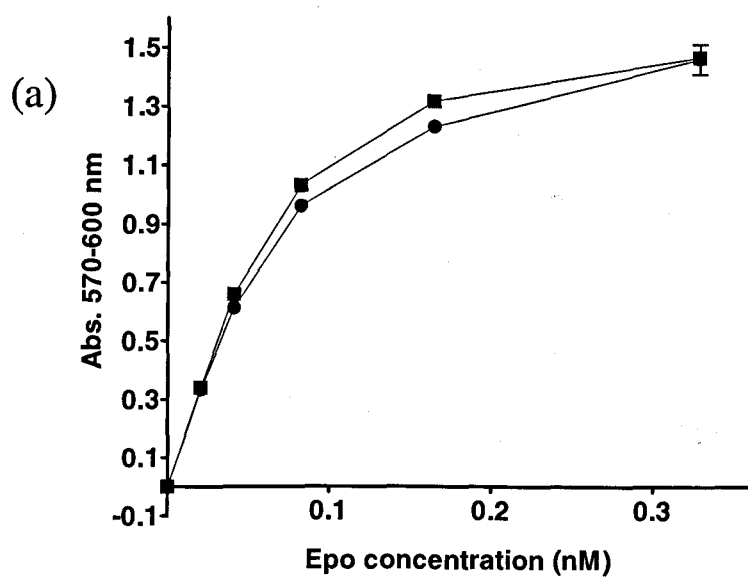
(c) AS-E2 cells transfected with the RCA<sub>120</sub>-encoding vector

(pIRESblast[RCA<sub>120</sub>-GH-R TM]).

Sample t=0 min (■) and sample t=1800 min (●). Data represent mean +/- s.e.m.

of triplicated samples (where an error bar is not shown, it lies within the dimensions of the symbol).





### **5.3.6. PCR-amplification from cell extracts**

Using vectors containing the IRES sequence between the gene of interest and the antibiotic-resistance gene was done to ensure that antibiotic-resistant cells contained genomic DNA for both genes from which mRNA would be transcribed. However, it was decided to verify that the gene for the chimeric lectin display protein had not been spontaneously deleted resulting in antibiotic-resistant cell lines containing only the antibiotic-resistant gene, something which could explain the antibiotic-resistance coupled with the lack of detectable chimeric protein expression. This was done by performing RCA B chain PCR-amplification (as in Section 2.1.3.2.) on cell extracts from empty pIRESblast- and pIRESblast[RCA<sub>120</sub>-Epo-R TM]-transfected cells prepared by proteinase K digestion (Section 2.2.3.4.).

A band of the expected size (~780 bp) was amplified by RCA-specific primers (primers RCA-5' and RCA-3' from Section 5.2.2.) from the pIRESblast[RCA<sub>120</sub>-Epo-R TM] vector (the positive control) and from the pIRESblast[RCA<sub>120</sub>-Epo-R TM]-transfected cell extract (Fig. 5.3.5.a). Bands were amplified by actin-specific primers (primers actin-5' and actin-3' from Section 2.2.3.4.) from the empty pIRESblast- and pIRESblast[RCA<sub>120</sub>-Epo-R TM]-transfected cell extracts but not from the water negative control (Fig. 5.3.5.b). This showed that both cell extracts contained PCR-amplifiable DNA and that the proteinase K digestion had worked. In conclusion, the pIRESblast[RCA<sub>120</sub>-Epo-R TM]-transfected cell line had not lost DNA amplifiable by primers specific for the RCA genes.

Fig. 5.3.5. (Facing page) PCR amplification of RCA (expected product size= $\sim$ 780 bp) and actin (expected product size= $\sim$ 630 bp) from cell extracts to determine whether the pIRESblast[RCA<sub>120</sub>-Epo-R TM]-transfected blasticidin-resistant cell line contained recombinant RCA B chain-amplifiable DNA.

(a) PCR amplification of RCA DNA using the RCA-5' and RCA-3' primers

(Section 5.2.2.). Ethidium Bromide-stained 1% agarose gel loaded as follows

(primers used at 20  $\mu$ M final concentration):

-Lane 1; primers RCA-5' and RCA-3' using pIRESblast[RCA120-Epo-R TM] vector DNA as a template (positive control).

-Lane 2; primers RCA-5' and RCA-3' using empty pIRESblast vector-transfected AS-E2 cell extract as a template.

-Lane 3; primers RCA-5' and RCA-3' using pIRESblast[RCA120-Epo-R TM]-transfected AS-E2 cell extract as a template

-Lane 4; primers RCA-5' and RCA-3' using sterile water as a template (negative control).

(b) PCR amplification of actin DNA using the actin-5' and actin-3' primers (Section

2.2.3.4.). Ethidium Bromide-stained 1% agarose gel loaded as follows (primers used at 2  $\mu$ M final concentration):

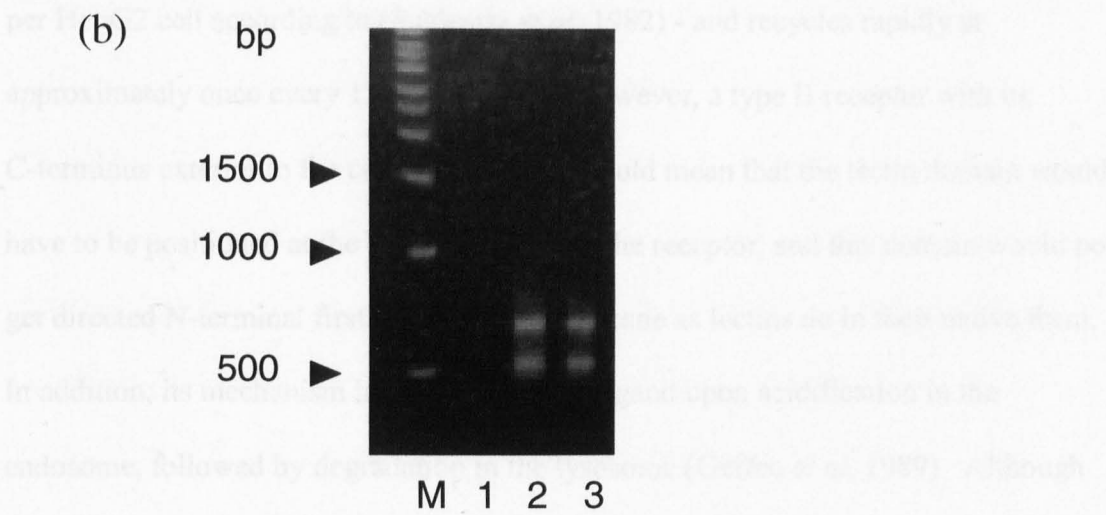
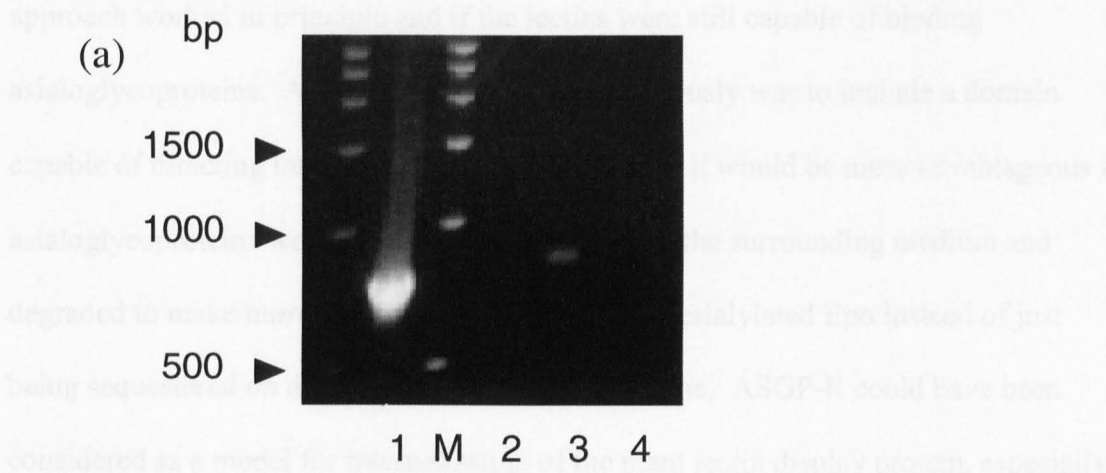
-Lane 1; primers actin-5' and actin-3' using sterile water as a template (negative control).

5.4. Discussion

(Fig. 5.3.5. continued)

-Lane 2; primers actin-5' and actin-3' using empty pIRESblast vector-transfected AS-E2 cell extract as a template.

-Lane 3; primers actin-5' and actin-3' using pIRESblast[RCA120-Epo-R TM]-transfected AS-E2 cell extract as a template.



## **5.4. Discussion**

Although any membrane-spanning sequence would in theory have been suitable for facilitating the display of galactose-binding plant lectins on the surface of an Epo-dependent cell line, that of Epo-R and its signal peptide were chosen because any processing machinery and accessory proteins involved in its being delivered to the surface of the cell would be present in AS-E2 cells.

The main objective was to display plant lectins on the cell surface to see if this approach worked in principle and if the lectins were still capable of binding asialoglycoproteins. A strategy explored simultaneously was to include a domain capable of directing internalisation and recycling, as it would be more advantageous if asialoglycoproteins were continually removed from the surrounding medium and degraded to make more binding sites available for desialylated Epo instead of just being sequestered on the surface of the cell membrane. ASGP-R could have been considered as a model for internalisation of the plant lectin display protein, especially as it does not transmit a proliferation signal, has high expression - 225,000 receptors per HepG2 cell according to (Schwartz *et al*, 1982) - and recycles rapidly at approximately once every 15 minutes. It is, however, a type II receptor with its C-terminus exterior to the cell, a fact which would mean that the lectin domain would have to be positioned at the C-terminal end of the receptor, and this domain would not get directed N-terminal first through the membrane as lectins do in their native form. In addition, its mechanism involves releasing ligand upon acidification in the endosome, followed by degradation in the lysosome (Geffen *et al*, 1989). Although *Ricinus communis* lectins (Frenoy *et al*, 1986; Frenoy, 1986) and lectins of the *Erythrina* genus (Konozy *et al*, 2003; Konozy *et al*, 2002) experience a decrease in affinity for ligand when the pH drops below neutrality, there was no guarantee that

asialo-Epo would dissociate from the plant lectin chimeric receptors to the same extent, thereby allowing unbound receptors to recycle back to the cell surface. It was therefore felt to be prudent to use the internalisation domain from the growth hormone receptor - a system that undergoes degradation of the ligand and its receptor. The Epo-R gets degraded but would not have been suitable as this degradation is inextricably linked with activation. Internalisation can take place irrespective of activation (Beckman *et al*, 1999) but the Epo-R fails to undergo degradation if activation is abrogated by tyrosine kinase inhibitors (Walrafen *et al*, 2005; Yang *et al*, 1996).

The growth hormone receptor, however, can undergo degradation independently of any signal transduction. When four prolines (residues 299, 300, 302 and 304) were changed to alanines within the rabbit GH-R's box-1 domain, Jak2 phosphorylation and activation were abolished, whereas ubiquitination and degradation proceeded normally (Alves dos Santos *et al*, 2001).

The RCA/GH-R chimeras incorporated residues 233 to 367 of the GH-R, since contained within them is the 10-amino acid ubiquitin-dependent endocytosis (Ube) motif Asp-Ser-Trp-Val-Glu-Phe-Iso-Glu-Leu-Asp (DSWVEFIELD), the ubiquitin-conjugation system being necessary for internalisation (van Kerkhof *et al*, 2000). The GH-R's Tyr- Glu-Gly-Phe-Ser (YEGFS) motif was retained as this corresponds to the stabilising Trp-Ser-Ala-Trp-Ser (WSAWS) motif in the Epo-R. Ligand-induced GH-R dimerisation may no longer have been possible by the GH-R-based chimeric receptor but although dimerisation is necessary for signal transduction, as shown using growth hormone mutated in such a way as to prevent two receptors from being bound at the same time, internalisation and degradation have been shown to still continue normally without it (Fuh *et al*, 1992; Harding *et al*, 1996).

Lectins form dimers and tetramers, most likely because the capacity for increased polyvalency promotes agglutination. The non-viability of the ECL-transfected cells may be a result of ECL's requirement for dimerisation. If results using either of the RCA lectins had been more promising, one option could have been to do some site-directed mutagenesis studies to try to make the ECL used in the ECL-TM display constructs monomeric, drawing inspiration from work by Narhi *et al* in which surface residues were changed to more charged residues thereby making them more stable and less likely to aggregate (2001). Cho *et al* (1996), in particular, made mutations in the gene for galaptin, another galactose-binding lectin, and succeeded in making the protein monomeric. The residues involved at the ECL dimer interface have been reported (Turton *et al*, 2004). Over half are apolar or polar rather than charged amino acids and could potentially be targeted by site-directed mutagenesis; the most likely candidates being isoleucine<sup>191</sup> and valine<sup>203</sup>.

Additionally, vector constructs could have been further optimised by replacement of the different regions. Transmembrane segments from different receptors could have been tried for this but maybe another option would have been to use one from an inhibitory receptor (although it is not known whether a negative signal could still be transmitted). Examples include receptors for tumour necrosis factor- $\alpha$ , interferon- $\gamma$ , or transforming growth factor- $\beta_1$ , all of which are powerful inhibitors of erythropoiesis (Zermati *et al*, 2000).

There are numerous stages at which things could have gone wrong: folding, insertion into the cell surface membrane, level of expression, or retention of galactose-binding activity. If it were decided to take this work further, the first step might be to establish whether the problem of lack of chimeric protein expression was at the DNA level or the protein expression level. Expression of RCA protein by AS-E2 cells could not be

detected by Western blot, despite the fact that in the other projects in which the vector pIRESblast was used, protein could be detected unless it was toxic to the cells, in which case no cells survived the blasticidin selection stages (Dr A. Macadam, personal communication, and other projects not described in this thesis). There was, however, the possibility that a potential toxicity of the lectin-containing genes could lead to selection of clones that have lost them. This was ruled out when the pIRESblast[RCA<sub>120</sub>-Epo-R TM]-transfected cell line was confirmed as containing DNA amplifiable by RCA-specific primers. Spontaneous point mutations could still have arisen, though, that introduced stop codons, resulting in truncated forms of the protein. Such types of mutations would need to be identified by sequencing. Toxicity of the lectin display proteins could be revealed by transiently transfecting the AS-E2 cell line with the empty pIRESblast vector or a lectin display protein-encoding vector and then counting live cell and dead cell numbers. Expressing the RCA<sub>60</sub> and RCA<sub>120</sub> lectin B chain domains on their own could be carried out to check that they can be synthesised in the AS-E2 human cell line. It is unlikely that this is a problem, however, as there are already reports of ricin being successfully expressed in mammalian (murine) cells (Krek *et al*, 1995). An alternative strategy would be to use the lectin display protein-encoding cDNA in an *in vitro* expression system to establish whether the proteins have the potential to be successfully synthesised in a different system and whether the lack of expression was specific to the mammalian expression system. If, in the future, RCA<sub>60/120</sub> were successfully expressed on the surface of cells, it may still not be effective without recycling, potentially an even more complicated objective to achieve.



## **5.5. Conclusion**

In conclusion, an attempt was made to genetically engineer the Epo-dependent AS-E2 cell line to express either the ECL or RCA<sub>60/120</sub> galactose-binding plant lectins at the extracellular surface of the cell. Cells stably transfected with an ECL-encoding vector did not survive and cells stably transfected with RCA<sub>60/120</sub>-encoding vectors gave negative results for RCA<sub>60/120</sub> by Western blot, indicating lack of expression. As expected from the lack of protein expression, cell-based assays using cells stably transfected with RCA<sub>60/120</sub>-encoding vectors, whether or not they contained an internalisation and degradation motif, failed to differentiate between intact and extensively desialylated Epo.

## **Chapter 6. Discussion**

### **6.1. Epo as a pharmaceutical analysis problem**

Epo has been on the market since 1989. In addition to its most commonly used role in stimulating proliferation of red blood cells, it has also been shown to be involved in regulating endothelial progenitor cells, neurones, myocardiocytes and vascular smooth muscle (Bahlmann *et al*, 2004; reviewed in Buemi *et al*, 2002):

- There is an Epo receptor on smooth muscle cells and endothelial cells which, when bound by ligand, can trigger the release of endothelin and inhibit nitric oxide synthase (nitric oxide being a vasodilator), resulting in contraction of the smooth muscle and vasoconstriction, and bringing about an increase in blood pressure (Buemi *et al*, 1993; Squadrito *et al*, 1999);
- Endothelial cells treated with Epo upregulate their numbers of vascular endothelial growth factor (VEGF), thereby potentiating proliferative effects and indicating a possible role in neovascularisation and wound-healing (Alvarez Arroyo *et al*, 1998);
- Epo-R is expressed early on in brain development and has a neuroprotective role, e.g. in combating cerebral ischaemia and concussive brain injury (Grasso *et al*, 2002) and preventing glutamate-induced cell death (Cerami *et al*, 2002). The brain makes its own erythropoietin but it is slightly smaller due to reduced sialylation, presumably because this form of Epo does not cross the blood brain barrier and come into contact with the general circulation where it could be cleared by the ASGP-R (Masuda *et al*, 1994).

Epo is no longer under patent (Schellekens, 2004) and many more companies are starting to manufacture it. It is therefore becoming increasingly urgent to find an appropriate test for Epo that does not use animals.

## **6.2. Control specifications for Epo**

The current approach to control of pharmaceutical Epo, as set out in the European Pharmacopoeia, specifies that only the normocythaemic or the post-hypoxic polycythaemic *in vivo* mouse bioassays can provide definitive information on biological activity as they alone take into account all factors (known and unknown) affecting activity.

The control of other glycoproteins (e.g. follicle-stimulating hormone (FSH), luteinising hormone (LH), thyroid-stimulating hormone (TSH) and human chorionic gonadotropin (hCG)) encounters the same problems in terms of the numbers of animals needed to test batches due to the wide variability of animal tests and consequently the large numbers of replicates required. For instance, it can take between 150 and 200 immature rats to obtain a statistically valid potency value for just one batch of FSH. Several hundred samples are submitted for testing each year, meaning that tens of thousands of animals are being killed annually (Driebergen & Baer, 2003).

Replacement assays would need to tackle the issues of structure-activity relationships and heterogeneity. There exists a complex relationship that is not yet fully understood between the carbohydrate structure of glycoproteins such as Epo and *in vivo* biological activity. It is difficult to directly translate information on a glycoprotein's carbohydrate structure into a prediction of its biological activity. The concept of purity cannot be applied in the case of glycoproteins as there is so much inherent

variability in the glycosylation process - there potentially exist hundreds of isoforms within a single batch.

### **6.3. Factors determining the *in vivo* biological activity of Epo**

In essence, *in vivo* activity is governed by a balance between the body's clearance mechanisms and Epo's affinity for binding its target receptor. As covered in Section 1.12., the factors reported to be involved include:

- Clearance mediated by the liver's asialoglycoprotein receptor of galactose-exposing glycoproteins;
- Clearance by the kidneys of glycoproteins containing bi-antennary structures;
- Receptor-mediated clearance.

### **6.4. Exposed galactosyl residues as a potential analytical target**

Analysis has shown that sialylation is the feature that varies most between batches of recombinant erythropoietin, while the sugar backbone structures remain the same – "the relative ratios of bi-antennary, tri-antennary, tetra-antennary and tetra-antennary saccharides with one, two or three N-acetyllactosaminyl repeats were almost identical among different samples" (Sasaki *et al*, 1987).

The first and subsequent Epo batches Sasaki *et al* analysed varied in terms of sialylation, as shown in the following compositions:

Level of sialylation	First batch	Other batches
Mono-sialylated	7%	Not reported
Di-sialylated	41%	18-21%
Tri-sialylated	48%	64-67%
Tetra-sialylated	4%	10-13%

This work was supported by LeFloch *et al* (2004) who removed sialic acids from recHuEpo, analysed oligosaccharides by high-performance capillary electrophoresis (HPCE) and showed that the profiles were very similar over the course of the batch culture, although if sialic acids were not removed then the profiles changed over the course of the culture and especially in the presence of serum.

While receptor-binding assays, for example, are generally acknowledged as not being sufficient to show whether a sample is biologically active, a receptor-binding assay may be "adequate for (quality control) if it has been shown to be sensitive to the variations known to occur in a growth factor preparation" (Robinson, 1999).

Similarly, for batch release, physico-chemical tests can be used to demonstrate that a biological is structurally identical to that which has been shown to be effective in clinical studies even though potency has not been demonstrated directly. It can be argued that since the factor which varies in the case of Epo (i.e. the level of sialylation) is known, assays can be designed with galactose as an analytical target in mind. This can be done physico-chemically (by glycan mapping and, to some extent, by IEF or other charge-based procedures) or by incorporating a galactose-dependent step or mechanism into *in vitro* bioassays.

#### **6.4.1. *In vitro* bioassays as analytical tools**

Cell-based bioassays were employed because they can be used to determine the biological activity of proteins in a way that physico-chemical methods, ELISAs and receptor-binding cannot. There is, in fact, resistance to replacing the *in vivo* mouse assay with purely physico-chemical methods. It is argued that although physico-chemical and biochemical tests are extremely good for analysing the precise molecular structural features of heterogeneous glycoproteins, it is not simply a question of translating such data into an assigned specific biological activity, and that only a bioassay can measure biological activity (Robinson, 1999). Cell-based bioassays take into account all aspects of biological activity; receptor binding, activation of signal transduction and the final biological outcome, e.g. proliferation, prevention of apoptosis, cell migration, differentiation etc (Mire-Sluis, 1999; Thorpe *et al*, 1997). Radioimmunoassays and ELISAs, for instance, are not completely reliable because they have the potential to also detect inactive precursors or breakdown products (Krystal, 1983). *In vivo* assays go one step further in that they are able to assess all aspects of a protein's biological activity, including glomerular filtration, clearance by the liver and metabolism by target organs.

Although undeniably sensitive, *in vivo* assays tend to be more labour-intensive and expensive than *in vitro* assays, and fail to provide the same level of precision. A considerable number of mice are frequently required in order to obtain a statistically valid potency value. They therefore tend to be employed only in cases such as that of glycoproteins when the parameters measured are not taken into account in the *in vitro* procedure. In addition to being more precise, immortalised, factor-dependent cell lines are more economical and easier to use and analyse (Thorpe *et al*, 1997), and

interassay variation is lower (Robinson, 1999). *In vitro* assays, such as those outlined in Chapters 3-5, which purely target exposed galactosyl residues would be unable to make a distinction between the following: completely deglycosylated Epo; Epo that is missing both sialic acid and galactose residues; fully sialylated bi-antennary Epo; and the ideal, fully sialylated, tetra-antennary Epo. Asialo- agalacto-Epo, however, could feasibly be depleted by agarose-conjugated succinylated wheat germ agglutinin which, according to reports, binds to N-acetylglucosamine, but not if it is sialylated (Monsigny *et al*, 1979). The properties of Concanavalin A might equally be exploited as it preferentially binds bi-antennary sugars (Treuheit *et al*, 1992).

#### **6.4.2. Incorporation of galactosyl-binding into the *in vitro* bioassay**

The studies in which neuraminidase- desialylated Epo is tested by the solid-phase AS-E2/ECL assay described in Chapter 3 provide proof of principle that this strategy can permit discrimination between differentially sialylated Epo samples.

An asset of the asialoglycoprotein receptor-expressing AS-E2 cell line bioassay of Chapter 4 is that it employs fewer manipulation steps compared with the AS/E2-ECL assay described in Chapter 3. For this reason, the same serially diluted Epo samples can be simultaneously compared in the genetically manipulated cell bioassays and in the unmodified Epo bioassay. Potential errors that might have arisen during serial dilution or as a result of degradation will thus be detected. This assay is not as sensitive, however, a problem that could be surmounted in three ways:

- (1) using a stronger galactose-binding lectin, i.e. certain plant lectins (which also have the advantage of not being so dependent on an oligosaccharide branch possessing three exposed galactose residues);

- (2) having a decoy receptor capable of internalisation, degradation and/or recycling;
- (3) increasing decoy receptor cell surface expression in order to compete with the high affinity of the Epo-R. This could be achieved by using the dihydrofolate reductase (DHFR) or glutamine synthetase (GS) genes to amplify the gene of interest, or making alterations to the signal peptide and transmembrane segment for optimal expression.

The third method, involving gene amplification, was attempted but problems arose during the vector ligation stages due to unknown reasons. A brief description follows of the intended strategy.

#### **6.4.2.1. Proposed strategy for increasing ASGP-R expression**

Gene amplification involves an increase in the copy number of a gene without a proportional increase in other genes in the genome (reviewed in Omasa, 2002). Two of the most common gene amplification systems are the DHFR and the GS amplification systems. A vector containing the amplification gene is introduced into a host cell line and then cells are grown in increasing concentrations of a drug that inhibits the gene – methotrexate in the case of the DHFR gene (Hakala, 1957) and methionine sulfoximine in the case of the GS gene (Sanders & Wilson, 1984). Cells are selected for containing increased copy numbers of the amplification gene. A gene of interest included in the vector alongside the amplification gene has the potential to be co-amplified. It has been proposed that the mechanism by which gene amplification takes place involves expansion of chromosome regions or chromosome breakage leading to the formation of extra chromosomal structures such as double



minute chromosomes (Paulson *et al*, 1998). An attempt was made to create a vector containing:

{[gene of interest]-[IRES]-[amplification gene]-[IRES]-[blastocidin resistance gene]}.

A disadvantage of DHFR-directed amplification is that it can only be used in DHFR-negative cells, whereas AS-E2 cells are DHFR-positive. This constraint can be avoided by modifying the DHFR gene by site-directed mutagenesis to contain an arginine instead of a leucine at position 22 (Simonsen & Levinson, 1983). The mutated version of DHFR is not able to bind its inhibitor methotrexate as strongly, and therefore much higher concentrations of methotrexate are needed before it gets inhibited and can direct gene amplification. Meanwhile, all endogenous DHFR activity has been completely abrogated by the higher methotrexate levels. The GS amplification system can be used in any cell type as well. Such a vector containing IRES sequences between the gene of interest, the amplification gene and the antibiotic-resistant gene would reduce the need to screen clones and would be more likely to ensure amplification of the gene of interest. A vector that contains the IRES sequence and the GS gene has been reported to enable selection "superior to a conventional expression vector in both levels of protein expression and amplification efficiency" (Pu *et al*, 1998).

There are also other, smaller changes that could be made that may increase expression. For example, the ASGP-R-H1 subunit has an atypical Kozak sequence and changing its ATCATGA sequence to ACCATGG may improve expression levels. The plant lectin display vector constructs could use the artificial "secrecon" signal sequence, as research has found this to be the best signal sequence for protein secretion (Barash *et al*, 2002) and it may also work for receptor expression.

If a receptor is introduced into a cell line with a view to conferring sensitivity to, for example, a cytokine, then high receptor-expression levels are not crucial as a response is still elicited. In the case of Epo, a half-maximal response in a cellular proliferation assay is produced at an Epo concentration of 0.01 nM, whereas half the Epo-R binding sites are bound at the higher concentration of 0.18 nM (Syed *et al*, 1998), prompting the authors to speculate that only 6% of receptors need to be bound for half-maximal proliferation. The aim of the research described in this thesis is a different one altogether and requires high levels of expression to sufficiently lower the concentration of the surrounding medium.

#### **6.4.2.2. Sialylation and *in vitro* activity**

An additional factor that must be taken into consideration is the increase in *in vitro* activity that has been reported when using desialylated Epo in comparison with fully sialylated Epo. This effect has been observed in bone marrow cells (Imai *et al*, 1990) and in the TF-1 cell line (Takeuchi *et al*, 1990) (three to fourfold increases being reported). Similar results were obtained using colony-forming assays; colony-forming unit (CFU) activity was 100% for intact fully sialylated Epo, decreased to 67% for deglycosylated (possibly due to lack of stability), but increased to 180% for desialylated Epo (Tsuda *et al*, 1990). In *in vivo* assays the latter two samples were devoid of activity. Such an effect was not observed to as significant a degree when using the unmodified AS-E2 bioassay. This is important because getting correlation with the *in vivo* assays using the assays outlined in this thesis would have been even more of a challenge if desialylated Epo samples had been several times more potent than a comparable concentration of intact Epo.

The difference in effect of sialylation on *in vitro* activity may be attributable to there being two known classes of Epo receptor: the high-affinity form has a  $K_d$  value of 0.08-0.1 nM for intact Epo, whereas the low-affinity form has a  $K_d$  value of 0.8-1.3 nM (Dong *et al*, 1992). By contrast, the  $K_d$  values for asialo-Epo are 0.02-0.03 nM and 0.9-1.2 nM respectively. The strength of interaction between the high-affinity receptor and Epo increases with desialylation, whereas desialylation does not appear to affect Epo binding to the low-affinity receptor. The AS-E2 cells used in the assays outlined in this thesis possess only the low-affinity receptor (Miyazaki *et al*, 1997). Accessory proteins may exist that confer high affinity to the Epo receptor. If CHO cells are fused with low-affinity receptor-expressing cells, the Epo receptor becomes a high-affinity one (Hermine *et al*, 1996) implying that CHO cells contain a protein or proteins that change the receptor's binding characteristics. The lower affinity of fully sialylated Epo is ascribed to the higher negative charge conferred by sialic acid (Imai *et al*, 1990), but how can this be reconciled with the fact that, as mentioned in Section 3.4., the sugars project some distance away from the receptor? Darling *et al* (2002) used surface plasmon resonance to show that sialic acids and carbohydrates in general inhibit the binding of Epo to isolated Epo-R. The isolated Epo-R was in the form of an Epo-R chimera bound to protein A and so was presumably free of any accessory proteins that could be causing the difference in the high-affinity binding cells. Long-range electrostatic forces were suggested to be involved as increasing the ionic strength reduced the rate of Epo binding to its receptor. This appears to contradict Dong *et al* (1992) who state that low-affinity Epo-R-expressing cells do not show a preference for asialo-Epo over fully sialylated Epo.

Schooley *et al* (1985) report an even greater rate of DNA synthesis if sialidase is included in the assay, leading the author to conclude that if it is accepted that desialylation allows Epo to gain easier access to its receptor by removing a large negative charge, then it could equally be argued that there is a negative charge on the surface of cells which can be removed by sialidase and which hinders receptor engagement.

Steinberg *et al* (1986) suggest that Epo produced when the body is under stress may be less well processed and consequently cleared more rapidly but would bind its receptor with more affinity. The case of follicle-stimulating hormone (FSH) may shed some light on the subject. FSH's state of glycosylation affects its activity, possibly allowing for a more finely tuned response to the hormone (Ulloa-Aguirre *et al*, 2003). Much like Epo, clearance of FSH is governed by the number of exposed galactose residues. Less sialylated FSH isoforms are released prior to ovulation, coinciding with a reduction in its half-life (Zambrano *et al*, 1995). Ulloa-Aguirre *et al* (2003) found that less acidic FSH isoforms elicited a faster response (induction of tPA enzyme activity in the ovaries of rats) compared with the more acidic form of the hormone and speculated that this could compensate for a relatively shorter half-life – "even a brief exposure of the target cell to short-lived but highly potent gonadotropin forms may be sufficient to evoke a biological response."

#### **6.4.2.3. The viability of targeting the galactosyl residue**

The approach of targeting galactose residues works only if the variation seen in Epo activity is of a kind that is caused solely by galactosyl exposure. Although the literature suggests that this is the case, the anion-exchange-separated fractions of sample set 3 did not conform to this prediction: all the fractions had fairly similar

numbers of exposed galactose residues (apart from Epo Fraction A which contained more) but the *in vivo* biological activity tended to increase, indicating some other factor was also playing a part in governing potency. Conversely, while Epo Fractions A and B differed in numbers of galactosyl residues exposed per glycan (0.457 compared to 0.404 respectively), their *in vivo* activity remained the same. Galactose exposure and *in vivo* activity did not correlate well (Pearson correlation  $R=-0.507$  for the normocythaemic assay). Sialic acid content correlated better (Pearson correlation  $R=0.930$ ), possibly suggesting that the presence of protective sialic acid caps was doing more than just preventing Epo from being cleared. Further investigation is needed into the possible causes before putting forward any predictions in terms of biological activity.

### **6.4.3. Non-biological approaches to identifying activity-determining features**

#### **6.4.3.1. The utility of charge-based separations**

Charge-based methods can show the range of isoforms relating to differentially sialylated variants. IEF and capillary zone electrophoresis have thus been assessed in terms of their ability to provide quantitative information on the distribution of Epo isoforms (Bristow & Charton, 1999). Mulders *et al* (1999) propose using IEF for also predicting follicle-stimulating hormone activity (FSH). An international collaborative study on the use of isoelectric focusing or capillary zone electrophoresis to predict the bioactivity of follicle-stimulating hormone (Storring *et al*, 2002) concluded that the work showed promise, the techniques concerned being precise and accurate enough to "distinguish between FSHs which might meet or fail European Pharmacopoeia requirements".

A variation suggested by Hermentin *et al* uses a hypothetical "Z number" to predict *in vivo* biological activity (1996, 2002). This is calculated by multiplying the proportion of each type of sialylated structure (tetra-sialylated, tri-sialylated, di-sialylated, mono-sialylated and asialylated), as determined by high performance anion exchange chromatography and pulsed amperometric detection (HPAEC-PAD), by the charge conferred by the corresponding number of sialic acids (4, 3, 2, 1, and 0 respectively).

The higher the Z number, the more biologically active was the batch of Epo.

These charge -based procedures do have some drawbacks, however, namely the fact that poly N-acetylactosamine ( $\text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 3$ ) repeats have, in addition to the number of exposed galactoses, been implicated in clearance by the ASGP-R (Fukuda *et al*, 1989). Such a permutation could be more difficult to detect on an IEF gel. The issue of differentiating between different levels of branching could be equally problematic.

#### 6.4.3.2. The utility of glycan mapping

Sugars affect activity and glycan mapping can provide extremely detailed information on carbohydrate structure; for example, on the percentage of tetrasialylated or tetra-antennary glycan content. Tetrasialylated glycan content was shown to correlate well with *in vivo* biological activity in the case of the pharmaceutical Epo preparations of sample set 2 (as reported in (Yuen *et al*, 2003)). In the case of the anion-exchange-separated fractions of sample set 3, both tetra-antennary and tetrasialylated content exhibited good correlations with activity measured by the normocythaemic assay - the majority having Pearson correlation coefficients higher than 0.9. It must be borne in mind, however, that the *in vivo* values involved in these

correlations were very closely grouped and did not differ from each other to any statistically significant extent.

Glycan mapping is, again, not a method that provides information on activity at the receptor and cellular level. Although it can provide an extremely detailed analysis of carbohydrate structure, the real challenge consists in fully interpreting that information.

There is a further complication in that, as mentioned in Section 1.2., glycoprotein oligosaccharide chains can vary immensely in terms of type of glycosylation (N- or O-type), number of branches, and level of sialylation, galactosylation, fucosylation and poly-lactosaminylation. A carbohydrate chain with any combination of these can occur at a glycosylation site. It is therefore exceedingly difficult to isolate and purify just one form. Accordingly, the precise roles assigned by the different oligosaccharide structures is by no means clear. A side-project was therefore undertaken to try to simplify matters by creating Epo that was sialylated at just one site. Such a protein would be easier to separate into its five fractions (1-4 sialic acids and one isoform without sialic acids). The five fractions could then be tested *in vivo* to see if there was a radical jump in activity between the tetra-sialylated form of Epo and all other forms (as suggested by the Yuen *et al* (2003) paper) or, alternatively, if the increase in activity was incremental and regular. Although beset with purification and concentration problems, that project is still ongoing.

Table 6.1. Methods available (including cell-based assays developed during the course of this project) for detecting changes in isoform distribution and how sensitively they behaved when testing each of the three Epo sample sets.

	Sample set 1 (neuraminidase-treated)	Sample set 2 (different culture and purification conditions)	Sample set 3 (ion-exchange fractionated pharmaceutical grade Epo)
<i>In vivo</i> assay	✓✓✓	✓✓	x
<i>In vitro</i> AS-E2 assay	x	x	x
<i>In vitro</i> AS-E2/ECL assay	✓✓	✓	x
Stably transfected ASGP-R expressing AS-E2 cells	✓	Not applied	Not applied
AS-E2 cells stably transfected with cell-surface displayed galactose-binding plant lectin	x	Not applied	Not applied
IEF	✓✓	✓✓	✓✓
Glycan mapping	Not applied	✓✓✓	✓✓✓



## **6.5. Conclusions, future developments and specification**

### **recommendations**

Table 6.1. summarises the power of the assays developed during the course of these studies as well as the techniques previously available to resolve differences in glycosylation within sample sets. Each method has its advantages and drawbacks. The following conclusions can be drawn in light of the information set out in the table:

1. The normocythaemic and post-hypoxic polycythaemic mouse assays are currently the only accepted procedure for assessing Epo biological activity. Although comprehensive, these assays present major drawbacks in that they require the use of animals, feature wide confidence intervals and are labour-intensive.
2. The cell-based bioassays offer ease of use, narrow confidence intervals and valuable information on biological activity at the target cell level. They suffer from a lack of sensitivity and the fact that they take into account only one carbohydrate structural feature (galactose exposure). The immobilised plant lectin assays appeared promising; although not as sensitive as *in vivo* assays, they revealed a statistically significant difference between intact Epo (Epo sample t=0 min) and slightly desialylated Epo (sample t=20 min) of sample set 1. The AS-E2 cells stably transfected with the ASGP-R were not as successful in this respect. In essence the problem is that although the most desialylated sample (t=1800 min) is detected as having a lower biological activity than does intact Epo in this cell bioassay, this variation between samples far exceeds what would be expected in recombinant batch production.

3. IEF is cheap, robust, not technologically demanding and provides good discrimination between different isoforms (this was the case in particular of the fractions in sample set 3 in Fig. 3.3.15.). If sialic acid content is a better indicator of activity, then IEF would be more appropriate than galactose exposure-dependent methods, as variation in pI is effected by sialic acids but not directly by galactose exposure. IEF does not go as far as glycan mapping in terms of the amount of detailed information provided, however.
4. Glycan mapping provides very detailed information about structure but it does not provide any information on activity of the protein in eliciting a response from its target cell. There are still uncertainties regarding sugar structure/activity relationships but at least information is provided on issues such as polylactosamine and bi-antennary content.

A good compromise may be to combine glycan mapping (for the most substantial analysis) or IEF (if an economic or technological trade-off is required) with an Epo-responsive cell-based bioassay. Continuing research into sugar structure-*in vivo* activity relationships would be necessary to enhance the interpretation of results obtained by glycan analysis.

## **Chapter 7. Summary**

Adequate glycosylation, in particular, sialylation, plays a critical role in the *in vivo* biological activity of Epo whereas it is mostly irrelevant to its *in vitro* activity.

Current *in vitro* ELISA- or cell-based assay results for Epo do not correlate with the *in vivo* bioassay results as the former assays do not take into account the body's various mechanisms for clearing the different glycoprotein isoforms, in particular, the role the liver plays in eliminating desialylated glycoproteins from the circulation. The aims of this project were to develop cell-based bioassays that were sialylation-sensitive and to assess their efficacy compared with the *in vivo* bioassays currently prescribed by the European Pharmacopoeia, as well as with an unmodified Epo-dependent cell line bioassay and two physico-chemical techniques (IEF and glycan mapping).

Three approaches were used to render cell-based bioassays sialylation-sensitive.

In the first approach, Epo was incubated with a solid-phase galactose-binding lectin (*Erythrina crista-galli*), mimicking passage through the liver *in vivo*, prior to serial dilutions of the Epo being used in a standard Epo-dependent cell line (AS-E2) bioassay. This assay was tested using Epo samples treated with neuraminidase for varying lengths of time. Incorporation of the ECL incubation step rendered the modified *in vitro* bioassay sensitive to desialylation, such that Epo desialylated almost to completion had less than 10% of the activity of untreated Epo. These results positively correlated with those obtained by *in vivo* assay in that *in vivo* activity was progressively abolished by desialylation through neuraminidase-treatment. *In vitro* activity, as measured by the unmodified AS-E2 assay, was essentially unaffected. In addition to the neuraminidase-treated Epo sample set, Epo produced by a range of different purification procedures (thus eliciting variation in terms of isoforms) and a

batch of pharmaceutical grade Epo separated by anion-exchange HPLC into fractions that varied by charge were also tested.

In the second approach to render cell-based bioassays sialyltation-sensitive, the AS-E2 cell line was stably transfected with the liver's asialoglycoprotein receptor, the receptor responsible for clearing glycoproteins containing galactose-terminated sugar chains. The objective was to introduce a receptor into an Epo-dependent cell line that would act as a decoy receptor, effectively sequestering and internalising Epo containing exposed galactose residues, and thus preventing such Epo molecules from binding the Epo receptor and transmitting a proliferation signal.

In the third approach, an attempt was made to stably transfect the AS-E2 cell line with a cell-surface-displayed plant-derived galactose-binding chimeric protein. The rationale behind this strategy was the same as that for the second approach except that the asialoglycoprotein receptor would be replaced by a plant lectin. Certain plant lectins have stronger binding affinities for galactose than the asialoglycoprotein receptor and their binding is not as dependent upon the oligosaccharide ligand having three terminal galactose residues.

These studies provided proof of principle that rational manipulation of *in vitro* bioassays could render these procedures responsive to factors known to affect bioactivity *in vivo*, and could therefore allow prediction of *in vivo* activity without using live animals.

Of the assays developed, the cell-based assay incorporating the immobilised-lectin binding step was the most successful strategy investigated. Although not as sensitive as the *in vivo* assay at detecting differences in potency, it did have narrower confidence intervals.

The asialoglycoprotein receptor-expressing Epo-dependent cell line assay was drastically less sensitive in terms of detecting differences in potency but it had the advantage of fewer handling steps and dilutions and could be directly compared with the untransfected cell bioassay.

It was not possible to test the hypothesis that plant lectin-displaying AS-E2 cells were more sensitive at detecting lower levels of desialylation or exhibited less of a bias towards tri-galactose terminating sugar chains, as there was serious doubt as to whether the cells successfully expressed the chimeric plant lectin:transmembrane domain protein.

The assays described in this thesis had the disadvantage that they take into account only one factor, namely the percentage of galactose exposure. Although it has been suggested that the latter is the main issue governing activity and the one most likely to vary in batch production, other factors are at play, possibly even some unknown ones. For instance, it has yet to be clarified to what extent the level of polylactosaminylation or the desialylated bi-antennary content may affect *in vivo* activity. Indeed, analysis carried out on pharmaceutical-grade charge-separated Epo fractions revealed that certain fractions differing in *in vivo* biological activity did not have varying levels of galactose exposure.

Replacing the animal test for Epo presents a considerable challenge, as it alone is able to take into account simultaneously the many factors affecting activity. Until we fully understand how sugars affect activity, designing an assay that can truly represent that activity will remain difficult. The attempts outlined in this thesis go some way to developing an *in vitro* cell-based test which, although it falls short in terms of sensitivity and does not take into account all aspects of sugars and multiple clearance mechanisms, does at least correlate with the *in vivo* assay.

Glycoanalysis appears to have the most potential for replacing the *in vivo* tests as it is extremely accurate and can assess all types of variation in oligosaccharide structure.

The best course of action, however, might be to recommend that it be combined with a cell-based bioassay, as the latter provides valuable information on whether the Epo protein structure is suitable for eliciting a proliferation response from its target cells.

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## Appendix

All reagents were obtained from Sigma unless otherwise stated.

All autoclaving was carried out for 22 minutes at 121°C, 1.05 bar.

### Referred to in Section 2.1.5.

*Recipe for Luria-Bertani (LB) medium:*

Reagent	Amount
Tryptone (Merck Biosciences, UK)	10 g
Yeast extract (BD Biosciences, UK)	5 g
NaCl (BDH Analar, UK)	5 g

MilliQ water was added to a final volume of 1 litre and autoclaved.

*Recipe for LBamp plates:*

Reagent	Amount
Tryptone (Merck Biosciences, UK)	10 g
Yeast extract (BD Biosciences, UK)	5 g
NaCl (BDH Analar, UK)	5 g
Agar (BD Biosciences, UK)	15 g

MilliQ water was added to a final volume of 1 litre and autoclaved. The solution was allowed to cool to ~50°C and ampicillin (Sigma, UK) added to a final concentration of 100 µg/ml. The medium (~20 ml) was poured into 90mm Petri dishes and allowed to cool, then stored at 4°C for up to four weeks.

### *LBamp plates with IPTG/X-Gal:*

LBamp plates were made as above then 100 µl of 100mM isopropyl-beta-D-thiogalactopyranoside (IPTG, Promega, UK) and 20 µl of 50 mg/ml 5-brom-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal, Promega, UK) were spread over their surface.

*Recipe for SOC (Super Optimal Broth with added glucose for catabolite repression) medium:*

Reagent	Amount
Tryptone	20g
Yeast extract	5g
NaCl	0.5

MilliQ water was added to a final volume of 1 litre and autoclaved. Filter-sterilised  $\text{MgCl}_2$  (1 M, 10 ml), filter-sterilised  $\text{MgSO}_4$  (1 M, 10 ml) and filter-sterilised glucose (20% [w/v], 20ml) were then added immediately before use.

### **Referred to in Section 2.1.7.**

*For 100 ml of Solution P1:*

Reagent	Mass/volume	Concentration
Glucose	0.9 g	50 mM
Tris/HCl (0.25 M) pH8.0	10 ml	25mM
EDTA (0.5M) pH8.0	2 ml	10mM

The volume was made up to 100 ml with MilliQ water, autoclaved and stored at 4°C.

*For 100 ml Solution P2:*

Reagent	Mass	Concentration/volume
NaOH	8 g	0.2 M
SDS	1 g	1%

The volume was made up to 100 ml with MilliQ water, filter-sterilised and stored at room temperature.

*For 100 ml Solution P3:*

Reagent	Amount/Volume	Concentration
Potassium acetate	29.442 g	3 M
Glacial acetic acid	11.5 ml	-

The volume was made up to 100 ml with MilliQ water, filter-sterilised and stored at 4°C.

**Referred to in Section 2.2.3.4.***Recipe for PBS used for washing cells:*

Reagent	Amount	Final concentration
Na <sub>2</sub> HPO <sub>4</sub>	1.44 g	0.01 M
KH <sub>2</sub> PO <sub>4</sub>	0.25 g	0.001 M
KCl	0.25 g	0.003 M
NaCl	10 g	0.171 M

The reagents were made up to 1 litre with MilliQ water.



**Referred to in Section 2.3.1.**

*Lysis buffer (6 ml total):*

Reagents	Initial concentration	Volume	Final concentration
Tris/HCl (pH 7.4)	200mM	600 µl	20 mM
NaCl	1.5M	600 µl	0.15M
Glycerol	100%	600 µl	10%
Triton-X-100	20% (v/v) in MilliQ water	300 µl	1%
EDTA	0.5 M	12 µl	1 mM
Leupeptin	100 µg/µl	60 µl	1 µg/µl
Phenylmethylsulphonylfluoride (PMSF)	200mM	30 µl	1mM

The volume was made up to 6 ml with MilliQ water.

**Referred to in Section 2.3.2.**

*SDS loading buffer (6x):*

Reagent	Concentration
Tris/HCl (pH 6.8)	0.35M
Sodium dodecyl sulphate	10.28% (w/v)
Glycerol	36% (v/v)
β-mercaptoethanol	5% (v/v)
Bromophenol blue	0.012% (w/v)

**Referred to in Section 2.3.3.***SDS running buffer:*

Reagent	Amount
Glycine	14.4 g
Tris	3.025 g
SDS	1 g

The volume was made up to 1 litre with MilliQ water.

**Referred to in Section 2.3.4.***Blot transfer buffer:*

Reagent	Amount
Glycine	14.4 g
Tris	3.0 g

The volume was first made up to 800 ml with MilliQ water and then up to 1 litre with methanol.

**Referred to in Section 2.3.7.***Recipe for Solution A (fixative):*

Reagent	Initial concentration	Volume	Final concentration
Formaldehyde (Sigma, UK))	37%	50 ml	5%
NaOH	4 M	0.2 ml	2 mM

The reagents were made up to 400 ml with MilliQ water and heated to 60°C.

*Recipe for Solution B (alkali accelerator):*

Reagent	Amount/volume
Anhydrous sodium carbonate	15 g
Formaldehyde (37%)	250 µl

The reagents were made up to 500 ml with MilliQ water.

**Referred to in Section 2.3.12.**

*Wash dilution buffer recipe:*

Reagents	Amount	Final concentration
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	1.95 g	2.5 mM
$\text{Na}_2\text{HPO}_4$	5.32 g	7.5 mM
NaCl	146.1 g	500 mM
Tween-20	5 ml	0.1% (v/v)

The reagents are made up to 5 litres with MilliQ water and the pH adjusted to 7.2 with NaOH. The Tween-20 is added after measuring the pH to avoid frothing during mixing. This buffer can be stored for 2-3 months at +4°C.

*Substrate buffer:*

Reagents	Amount	Final concentration
Citric acid. $\text{H}_2\text{O}$	7.3 g	34.7 mM
$\text{Na}_2\text{HPO}_4$	9.47 g	66.7 mM

The reagents are made up to 1 litre with MilliQ water, and the pH adjusted to 5.0 with NaOH. This solution can be stored for up to 2 months at +4°C.

**Referred to in Section 2.4.**

*Recipe for Albumin-buffered saline solution pH7.2 (ABS):*

Reagent	Amount
NaCl	6.95 g
NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	0.851 g
Na <sub>2</sub> HPO <sub>4</sub>	3.097
MilliQ	850 ml
EDTA solution (7% [w/v] in MilliQ water)	9 ml
Merthiolate (0.1% [w/v] in 0.9% [w/v] NaCl solution)	90 ml
Albumin	1 g

The pH of the solution was adjusted to 7.2 using NaOH or HCl. The volume was then made up to 1 litre with MilliQ water.

*Recipe for citrate buffer (50 ml):*

Reagent	Amount
Sodium citrate	500 mg
NaCl	295 mg
MilliQ water	40 ml

The pH was adjusted to 7.0 and the volume made up to 50 ml with MilliQ water.